Remifentanil preconditioning alleviates myocardial ischemia/reperfusion injury in rats via activating Jagged-1/Notch signaling pathway

Dejun Cao¹,#, Shaoxing Liu¹,#, Mengchang Yang², Keyu Xie¹, Zhuo Zheng¹, Hucheng Wen¹, Xianfeng Xie¹,*

¹ Department of Anesthesiology, Chengdu Second People’s Hospital, No. 10 Qingyun South Street, Chengdu 610000, China.
² Department of Anesthesiology, Sichuan Provincial People’s Hospital, No. 32 West Section 2, First Ring Road, Chengdu 610000, China.

# These authors contribute equally to this work.

* Correspondence to: Xianfeng Xie
Department of Anesthesiology, Chengdu Second People’s Hospital, No. 10 Qingyun South Street, Chengdu 610000, China.
Email: gangpan59834@163.com.
Tel: 028-86747892.

Short title: Cardioprotective effects of remifentanil preconditioning
Abstract: Ischemic heart diseases have emerged as great threats to human health. Nowadays, restoration of cardiac blood flow supply is widely regarded as a feasible treatment choice for ischemic heart diseases; however, this intervention would contradictorily elicit reperfusion injury. Recently, myocardial ischemia/reperfusion injury (MI/RI) has aroused widespread public concerns. Remifentanil, an ultra-short acting opioid analgesic, is frequently used for surgical anesthesia. Previous studies have demonstrated the cardioprotective effects of remifentanil preconditioning in clinical practice and in vitro experimental models; however, its exact mechanisms remain largely unclear. This study aimed to further evaluate the protective effects of remifentanil preconditioning against MI/RI and elucidate the potential molecular mechanisms. Rat models of MI/RI were successfully established via ligation of left anterior descending coronary artery for 30 minutes and restoration of blood flow for 2 hours. Herein, animal experiments displayed that remifentanil preconditioning could alleviate myocardial damage in rat models of MI/RI. Consistently, cell model experiments implied that remifentanil preconditioning attenuated hypoxia/reoxygenation exposure-induced injury in rat cardiomyocytes. Moreover, our findings verified the involvement of Notch signaling pathway in the protective effects of remifentanil preconditioning. In addition, mechanistic studies revealed that remifentanil preconditioning could up-regulate Jagged-1 expression and that Jagged-1 mediated the cardioprotective effects of remifentanil preconditioning through activating Notch signaling pathway. Taken together, our data indicate that remifentanil preconditioning ameliorates myocardial damage in rat MI/RI models via Jagged-1-mediated Notch signaling pathway activation. Thus, this study may offer some novel clues for understanding the cardioprotective mechanisms of remifentanil.
preconditioning against MI/RI.

**Keywords:** remifentanil preconditioning, myocardial ischemia/reperfusion injury, Jagged-1, Notch signaling pathway, rats

**Introduction**

In recent decades, ischemic heart diseases have emerged as great threats to human health all over the world [1-3]. It is reported that a vast number of patients are suffering from ischemic heart diseases, which brings huge pressures on living quality [4, 5]. Furthermore, it is well known that myocardial ischemia is closely associated with morbidity and mortality of patients with coronary artery disease and acute myocardial infarction [6, 7]. Nowadays, immediate restoration of cardiac blood flow supply is widely considered as a feasible clinical practice to ameliorate ischemic heart diseases; however, this intervention usually brings some unwanted side effects and would paradoxically elicit reperfusion injury [8-10].

It is widely acknowledged that myocardial ischemia/reperfusion injury (MI/RI) may facilitate the deterioration of cardiac functions via suppressing ventricular contraction as well as triggering arrhythmia, stroke and heart failure [11-13]. Hence, there is an urgent demand to develop effective pharmacological agents and unveil the potential mechanisms underlying the cardioprotective effects. Furthermore, mounting evidence has manifested that ischemic preconditioning is very beneficial to mitigating MI/RI in mice and rats, which could restrain infarction size, inhibit oxidative stress, enhance cardiomyocyte viability, and avoid inducing lethal arrhythmia [14-17]. It is well documented that remifentanil, a fentanyl derivative, could be rapidly metabolized and degraded by nonspecific esterases in the blood and tissues, making it attractive for intraoperative titration and fast recovery [18, 19].
Nowadays, remifentanil is frequently used as an ultra-short acting opioid analgesic in surgical anesthesia due to its unique pharmacokinetic characteristics [20, 21]. Notably, previous researches have demonstrated that remifentanil preconditioning exhibits a powerful cardioprotective capability in clinical practice and in vitro experimental models [22-26]. Nonetheless, the exact mechanisms underlying the cardioprotective effects are still not fully understood.

The current study aimed to further evaluate the cardioprotective effects of remifentanil preconditioning and illuminate the potential molecular mechanisms involved. Herein, we investigated the impacts of remifentanil preconditioning in both experimental rat models and hypoxia/reoxygenation-exposed cardiomyocytes, and assessed the possible involvement of Notch signaling pathway in the cardioprotective effects. Collectively, our findings indicate that remifentanil preconditioning attenuates MI/RI in rats via Jagged-1-mediated activation of Notch signaling pathway. Hence, the present study may offer some novel clues for understanding the mechanisms underlying the cardioprotective effects of remifentanil preconditioning.

Materials and methods

Animals

Male Sprague-Dawley rats (8 weeks of age, eweighing 250-300 g) were purchased from Shanghai Laboratory Animal Center (Shanghai, China). All the rats were fed water and standard chow ad libitum and housed in separated cages under a 12h light/dark cycle at 22 ± 2 °C. Animal experiments were performed in Laboratory Animal Center of Chengdu Second People’s Hospital (Chengdu, China).

Rat MI/RI model establishment

Rat MI/RI models were established as previously described. In brief, rats were
fixed on an operation table after being anesthetized via an intraperitoneal injection of 2% pentobarbital sodium. Absence of corneal reflex was considered as an indicator of successful anesthesia. The hearts were exposed and a suture was passed around the left anterior descending (LAD) coronary artery. The LAD coronary was occluded for 30 min through tightening the snare, followed by 2 hours' blood flow restoration. As for the sham model, a suture was placed around the LAD coronary without ligation.

**Adeno-associated virus vector (AAV) construction**

Recombinant adeno-associated virus vectors carrying Jagged-1 (AAV-Jagged-1) were constructed by Shanghai GeneChem Co. Ltd (Shanghai, China). Besides, adeno-associated virus vectors carrying green fluorescent protein (AAV-Con) served as the negative control.

**Animal experimental design**

This study was approved by Experimental Animal Ethics Committee of Chengdu Second People’s Hospital (IRB approval No. CDSPH201901070215). Animal experiments were performed in Laboratory Animal Center of Chengdu Second People’s Hospital (Chengdu, China). Animal experimental protocols conformed to the National Institutes of Health (NIH) Guidelines for Laboratory Animal Use and Care. In the current study, two animal experiments were conducted to evaluate the impact of remifentanil preconditioning and Jagged-1 over-expression on MI/RI in rats, respectively. In the first animal experiments, rats were randomly classified into three experimental groups (n=4): (i) sham group; (ii) I/R group; (iii) I/R+RPC group. For I/R+RPC group, rats were subjected to 3 consecutive intravenous injection of remifentanil (6 μg/kg) interspersed with 5-min infusion-free periods before I/R
treatment. As for the sham group and I/R group, rats were given normal saline. In the second animal experiment, rats were randomly assigned into 4 experimental groups (n=4): (i) sham group; (ii) I/R group; (iii) I/R+AAV-Con; (iv) I/R+AAV-Jagged-1. For I/R+AAV-Con group and I/R+AAV-Jagged-1 group, rats were administrated 100 μl of AAV-Con and AAV-Jagged-1 (5 × 10⁹ PFU/mL) via tail intravenous injection in 72 hours before I/R treatment, respectively. At the end of animal experiments, rats were intraperitoneally injected with 2% pentobarbital sodium for euthanasia, then blood and heart samples were collected for subsequent analysis.

**Measurement of myocardial infarction size via TTC staining**

Myocardial infarction size was evaluated via 2,3,5-triphenyltetrazolium chloride (TTC) staining. Briefly, the hearts were trimmed out and immersed in 10% potassium chloride solution. Then the hearts were frozen at -20 °C for 30 min, and sectioned into 2 mm-thick slices from apex to base. The slices were incubated with 1% TTC (Solarbio, Beijing, China) in the dark for 30 min at 37 °C and fixed in 10% formaldehyde for 24 h. Images were captured via a Nikon Eclipse TE2000 digital camera (Nikon, Tokyo, Japan). Infarcted area (pale white) and non-infarcted area (brick red) were analyzed using Image Pro v6.0 software (Media Cybernetics, Bethesda, MD, USA). Myocardial infarction size was expressed as the percentage of infarcted area/total area.

**Lactate dehydrogenase (LDH) activity assay**

Activity of LDH in coronary effluent was assessed via the colorimetric method using the LDH assay kit (Nanjing Jiancheng Bioengineering Co., Nanjing, China) in accordance with the manufacturer’s instructions. LDH activity was presented as unit per liter (U/L).
Detection of hypoxia-inducible factor-1 (HIF-1)

Cardiac tissues were prepared as 10% (w/v) homogenates in phosphate buffer saline (PBS) solution (100 mM, pH 7.4). HIF-1 levels in the homogenates were measured using ELISA kit (Shanghai Keshun Biotech Co., Shanghai, China) according to the manufacturer’s protocols.

Western blotting analysis

Total proteins were extracted in a RIPA buffer containing proteinase inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA). The BCA Protein Assay Kit (Beyotime, Beijing, China) was used to determine the concentrations of extracted proteins according to the manufacturer’s instructions. Total protein samples (20 μg) were separated on the SDS-PAGE gels and transferred onto the PVDF membranes. The PVDF membranes were sealed using 10% non-fat milk for 2 h and then incubated with primary antibodies: anti-Jagged-1 (1:1000, #7771, Abcam, Cambridge, MA, USA), anti-Jagged-2 (1:1000, #10556, Abcam), anti-NICD (1:1000, #167441, Abcam), anti-Hes1 (1:1000, #221788, Abcam), anti-cleaved caspase 3 (1:1000, #9661, CST Biotech, Beverly, MA, USA), anti-Bcl2 (1:1000, #194583, Abcam) and anti-β-actin (1:1000, #6276, Abcam) at 4 °C overnight. Subsequently, the membranes were incubated with horseradish peroxide (HRP)-labeled secondary antibody for 1 h at room temperature. The enhanced ECL kit Pierce Biotech, Rockford, IL, USA) was used to visualize the blot bands.

Quantitative PCR (qPCR) analysis

Total RNA was extracted via TRIZol reagent (TaKaRa, Dalian, China) according to the manufacturer’s protocols. The RNA samples were then reverse-transcribed using the M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Amplification was
conducted by the SYBR Green qPCR Master Mix (TaKara) on an ABI Prism 7500 PCR System (Applied Biosystems, Foster City, CA, USA). The primers were synthesized by Shanghai Sangon Biotech Co., Ltd (Shanghai, China). The information concerning primers was listed as followed: 5’-CATCGATGAATGTGCCAGCA-3’ (forward) and 5’-GCAGTGGTCTTTCAGGTGTG-3’ (reverse) for Jagged-1; 5’-GTCAAGGTGGAGACGGTTGT-3’ (forward) and 5’-TGGTAGAGCAGCTTTGTG-3’ (reverse) for Jagged-2; 5’-GGAGATTACTGCCCTGGCTCCT-3’ (forward) and 5’-GGCCGGACTCATCGTACTCCTG-3’ (reverse) for β-actin. The cycling parameters were provided below: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 sec, 60 °C for 1 min and 72 °C for 30 sec. Results were calculated using the 2^{-△△Ct} formula and normalized to β-actin.

**Cell culture and cell transfection**

Rat H9c2 cardiomyocytes were obtained from ATCC (Manassas, VA, USA) and maintained in DMEM supplemented with 10% fetal bovine serum (FBS) in humidified incubator under a 5% CO2 atmosphere at 37 °C. Cell transfection was carried out using Lipofectamine 2000 (Invitrogen) in line with the manufacturer’s instructions. Jagged-1 over-expression vectors were constructed via inserting Jagged-1 fragment into pcDNA3.1 plasmids by Shanghai GeneChem Co. Ltd. Besides, the empty vector pcDNA3.1 plasmids were used as a negative control. Transfection efficiency was evaluated by Western blotting at 48 h post-transfection.

**In vitro hypoxia/reoxygenation (H/R) experiments**

In order to induce I/R injury, H9c2 cells were incubated in hypoxic medium and exposed to hypoxic conditions (95% N2 + 5% CO2) provided by a hypoxia chamber
(StemCell Technologies, Vancouver, BC, Canada) for 90 min. The cells were then changed to normal culture conditions (95% O2 + 5% CO2) and maintained for 120 min. As for remifentanil preconditioning, H9c2 cells were pre-incubated with remifentanil (Toronto Research Chemicals; Toronto, Ontario, Canada) for 10 min before H/R treatment.

**Cell viability assay**

Cell viability was detected using the Cell Counting Kit-8 (CCK8; Dojindo, Kumamoto, Japan) according to the manufacturer’s instructions. In brief, H9c2 cardiomyocytes were seeded in 96-well plates at the concentration of 1 × 10⁴ cells/well. After hypoxia/reoxygenation exposure, cells were then incubated with CCK8 solutions for 4 h. The absorbance was measured at 570 nm via a microplate reader.

**Assessment of cell apoptosis**

Cell apoptosis was examined by the Annexin V-FITC/PI Apoptosis Detection Kit (Roche Diagnostics) based on the manufacturer’s protocols. Briefly, cells were gently resuspended in binding buffer containing Annexin V-FITC and PI after being washed with phosphate buffer saline (PBS) twice. Subsequently, cells were incubated for 15 minutes in the dark at room temperature. Stained cells were then analyzed via a flow cytometer (BD Biosciences, San Jose, CA, USA).

**Detection of intracellular reactive oxygen species (ROS)**

Intracellular ROS level was measured using the dichlorofluorescein diacetate (DCFH-DA; Sigma, Saint Louis, MO, USA) assay. In brief, H9c2 cardiomyocytes were incubated with the fluorescence probe of DCFH-DA for 20 min at 37 °C. Then the fluorescence of stained cells was determined using the FACSCalibur Flow Cytometry.
Statistical analysis

Experimental data were expressed as the mean ± standard deviation. Statistical analysis was conducted via SPSS 22.0 software (SPSS Inc., Chicago, IL, USA). Differences among three or more groups were evaluated using one-way analysis of variance (ANOVA) and Duncan’s post-hoc test. Differences were considered to be statistically significant when $P$-value < 0.05.

Results

Remifentanil preconditioning alleviates myocardial damage and activates Notch signaling pathway in rat models of MI/RI

Herein, MI/RI models were successfully established via ligation of left anterior descending coronary for 30 minutes and subsequent restoration of blood flow for two hours (Figure 1A and 1B). Firstly, we investigated the effects of remifentanil preconditioning on MI/RI in rat models. As shown in Figure 1A, higher LDH activity was observed in the coronary effluent of rats from the I/R group compared with the sham group; remifentanil preconditioning significantly reversed the increase in LDH activity triggered by ischemia/reperfusion (I/R) treatment. Furthermore, TTC staining demonstrated that remifentanil preconditioning remarkably mitigated the myocardial infarction of I/R-treated rats (Figure 1B), suggesting the protective effects of remifentanil preconditioning against I/R-induced myocardial damage. Furthermore, the levels of HIF-1 in the cardiac tissues were determined before and after remifentanil preconditioning. Notably, remifentanil preconditioning triggered a significant increase in HIF-1 level (Figure 1C). Subsequently, the involvement of Notch signaling pathway in the alleviating effects of remifentanil preconditioning was
evaluated via Western blotting analysis. As evident from Figure 1D, remifentanil preconditioning led to a dramatic increase in the expression levels of NICD and Hes1 proteins, indicating the activation of Notch signaling pathway. Taken together, the above findings imply that remifentanil preconditioning ameliorates myocardial damage and activates Notch signaling pathway in rat MI/RI models.

**Remifentanil preconditioning promotes Jagged-1 expression in damaged rat myocardial tissues and cardiomyocytes**

In order to further evaluate the possible involvement of Notch signaling pathway in the protective effects of remifentanil preconditioning, the expression patterns of its two crucial ligands, namely Jagged-1 and Jagged-2, were determined using qPCR analysis and Western blotting. As presented in Figure 2A and 2B, remifentanil preconditioning triggered a notable increase in Jagged-1 mRNA and protein expression level; however, no significant differences about Jagged-2 mRNA and protein expression levels were observed in the myocardial tissues among the sham group, I/R group and RPC+I/R group. Moreover, in vitro experimental models were constructed via hypoxia/reoxygenation (H/R) treatment in H9c2 rat cardiomyocytes. Afterwards, we further evaluated the effects of four various concentrations (0.1, 1, 2 and 5 μM) of remifentanil preconditioning on the protein expression of Notch ligand Jagged-1 in H/R-treated H9c2 rat cardiomyocytes. Western blotting analysis demonstrated that remifentanil preconditioning at the concentration of 1, 2 or 5 μM significantly facilitated Jagged-1 protein expression (Figure 2C). Notably, preconditioning of 2 μM remifentanil exhibited highest promoting effect on Jagged-1 protein expression in H/R-challenged H9c2 cardiomyocytes, and was thereby chosen for usage in the following cell experiment.
(Figure 2C). To sum up, these results suggest that remifentanil preconditioning facilitates Jagged-1 expression in damaged rat myocardial tissues and cardiomyocytes.

**Remifentanil preconditioning ameliorates H/R-induced injury in rat cardiomyocytes**

Herein, we explored whether remifentanil preconditioning could exert protective effects against H/R-induced injury in an in-vitro cell model. As determined by CCK8 assays, remifentanil preconditioning dramatically ameliorated the decrease in H9c2 cell viability induced by H/R treatment (Figure 3A). As shown in Figure 3B, remifentanil preconditioning triggered a notable decrease in ROS accumulation in H9c2 cardiomyocytes in comparison with the H/R group. Furthermore, a much lower proportion of apoptotic H9c2 cells was observed in the RPC+H/R group compared with that in the H/R group (Figure 3C). Western blotting analysis revealed that a lower cleaved caspase-3 expression level and a higher Bcl2 expression level were found in the RPC+H/R group in comparison with the H/R group (Figure 3D), which was consistent with the results about flow cytometry (Figure 3B). In addition, the effects of remifentanil preconditioning on Jagged-1/Notch signaling pathway in H/R-challenged H9c2 cardiomyocytes were also evaluated. As illustrated in Figure 3D, remifentanil preconditioning markedly elevated the protein expression levels of Jagged-1, NICD and Hes1 in H/R-treated cardiomyocytes, indicating the activation of Jagged-1/Notch signaling pathway. Collectively, the above findings demonstrated that remifentanil preconditioning could relieve H/R-induced damage in rat cardiomyocytes.

**Jagged-1 over-expression attenuates the damage of H/R-challenged rat cardiomyocytes**
On the basis of the findings mentioned above, it was speculated that Jagged-1 may serve as a crucial player in mediating the cardioprotective effects of remifentanil preconditioning. In order to elucidate the potential molecular mechanisms underlying the protective effects of remifentanil preconditioning, we then evaluated the impact of Jagged-1 over-expression on H/R-challenged H9c2 cardiomyocytes. As presented in Figure 4A and 4C, Jagged-1 over-expression significantly enhanced the viability of H/R-treated H9c2 cardiomyocytes and suppressed their apoptosis. Western blotting analysis showed that Jagged-1 over-expression notably repressed cleaved caspase-3 protein expression and facilitated Bcl2 protein expression (Figure 4D). Furthermore, it was found that Jagged-1 over-expression dramatically reduced the accumulation of ROS in H9c2 cardiomyocytes (Figure 4B). Besides, Western blotting analysis displayed that Jagged-1 over-expression promoted the activation of its downstream Notch signaling pathway (Figure 4D). To summarize, these results imply that Jagged-1 over-expression attenuates the damage of H/R-challenged rat cardiomyocytes, suggesting that Jagged-1 over-expression exhibits similar cardioprotective effects with remifentanil preconditioning.

**Inhibition of Notch signaling pathway destroys the protective effects of remifentanil preconditioning against H/R-induced cardiomyocyte injury**

With the purpose of further clarifying the possible mechanisms underlying the cardioprotective effects of remifentanil preconditioning, the influence of specific inhibition of Notch signaling pathway on H/R-exposed H9c2 cardiomyocytes was assessed. As evident from CCK8 assays, specific inhibition of Notch signaling pathway significantly reversed the promoting effects of remifentanil preconditioning on H/R-exposed H9c2 cell viability (Figure 5A). As presented in Figure 5B and 5C,
specific blockade of Notch signaling pathway remarkably abrogated the suppressing effects of remifentanil preconditioning on ROS accumulation and apoptosis in H/R-challenged H9c2 cardiomyocytes. To sum up, these findings suggest that inhibition of Notch signaling pathway destroys the protective effects of remifentanil preconditioning against H/R-induced H9c2 cardiomyocyte injury.

**Jagged-1 over-expression exerts protective effects against myocardial injury in rat models**

In rat MI/RI models, we explored the impact of Jagged-1 over-expression on damage. As displayed in Figure 6A, Jagged-1 over-expression dramatically attenuated the increase in LDH activity of the coronary effluent from I/R-challenged rats. Besides, TTC staining showed that Jagged-1 over-expression significantly alleviated the myocardial infarction of I/R-treated rats (Figure 6B). Additionally, Western blotting analysis demonstrated that Jagged-1 over-expression markedly elevated the expression levels of NICD and Hes1 proteins in the myocardial tissues of I/R-challenged rats, suggesting the activation of Notch signaling pathway (Figure 6C). Taken together, these results indicate that Jagged-1 over-expression exerts protective effects against myocardial damage in rat models, which is in consistency with the findings in vitro. Furthermore, the key findings that remifentanil preconditioning attenuates MI/RI in rats via activating Jagged-1/Notch axis have also been summarized in the proposed schematic presentation (Figure 7).

**Discussion**

In recent decades, ischemic heart diseases have already become increasingly prevalent worldwide and have imposes heavy burdens on public health [27, 28]. It is widely acknowledged that ischemic heart diseases remain one of the leading causes
of cardiovascular disorder-related deaths all around the world. Up to now, restoration of cardiac blood flow supply is still a routine treatment approach for myocardial ischemia; nevertheless, this reperfusion therapy strategy usually results in some severe adverse effects, such as stroke, ventricular contraction dysfunction, and myocardial infarction [29, 30]. In addition, accumulating studies have demonstrated that ischemic preconditioning could effectively ameliorate MI/RI in experimental rat models. Zhang et al. found that dexmedetomidine preconditioning attenuated MI/RI in rats via inhibiting the HMGB1/TLR4/MyD88/NF-κB pathway [14]. Liang et al. revealed that coenzyme Q10 preconditioning alleviated MI/RI and improved cardiac function in rats through regulation of oxidative stress and myocardial apoptosis [15]. Therefore, it is of vital necessity to develop effective ischemic preconditioning measures for cardioprotection and illustrate the relevant action mechanisms.

It is worth noting that remifentanil preconditioning has been demonstrated to display potent cardioprotective activity and improve heart function in both clinical application and experimental studies [22, 23, 26]. Nevertheless, the exact molecular mechanisms for the cardioprotective effects of remifentanil preconditioning remain largely unclear. Thus, the present study further investigated the influences of remifentanil preconditioning in both experimental rat models and hypoxia/reoxygenation-challenged H9c2 cardiomyocytes. Herein, rat MI/RI models were successfully established via 30 minutes’ ligation of left anterior descending coronary artery and subsequent two hours’ blood flow restoration. It is well acknowledged that heart damage is usually featured by myocardial infarction and cytoplasmic LDH release [31, 32]. Moreover, it is widely acknowledged that oxidative
stress and cardiomyocyte apoptosis are crucial biological events during the process of MI/RI development [33-36]. Based on rat model experiments, it was found that remifentanil preconditioning remarkably alleviated the increase in myocardial infarction size and cytoplasmic LDH release induced by I/R treatment. Furthermore, remifentanil preconditioning led to a remarkable increase in HIF-1 level the cardiac tissues. Notably, recent studies have revealed that elevated HIF-1 could protect the organs from I/R-induced damage through potentiating antioxidant activity and facilitating cell survival [37-39]. Besides, cell model experiments showed that remifentanil preconditioning significantly reversed the increase in intracellular ROS accumulation and apoptosis of H9c2 cardiomyocytes triggered by hypoxia/reoxygenation exposure. Taken together, our in vivo and in vitro experiments manifested the cardioprotective effects of remifentanil preconditioning.

It is well documented that Notch signaling pathway is a highly evolutionally conserved intercellular communication mechanism, which plays important roles not only in embryonic development but also in inflammation, cell differentiation, cell fate decision and cell apoptosis [40, 41]. Furthermore, accumulating researches have implied that activation of Notch signaling pathway would facilitate organ protection and improve heart function via repressing oxidative stress and enhancing cell survival [42-45]. In order to illustrate the potential molecular mechanisms by which remifentanil preconditioning exerted its cardioprotective effects, we assessed the possible involvement of Notch signaling pathway. It was observed that remifentanil preconditioning promoted activation of Notch signaling pathway through up-regulating its ligand Jagged-1. Moreover, cell model experiments and animal model experiments consistently revealed that Jagged-1 over-expression displayed
similar cardioprotective effects to remifentanil preconditioning. Based on the above findings, it is implied that remifentanil preconditioning alleviates MI/RI in rats partially via Jagged-1-mediated Notch signaling pathway activation. Undoubtedly, there are some limitations about unveiling the deeper molecular mechanisms underlying the cardioprotective effects of remifentanil preconditioning in the current study due to restricted experimental conditions. Thus, more efforts would be focused on further elaborating the exact molecular mechanisms in our future researches, especially exploring the possible crosstalk between Notch and NF-κB crosstalk.

In summary, the present study demonstrates for the first time that remifentanil preconditioning promotes activation of Notch signaling pathway via up-regulating its ligand Jagged-1 and that remifentanil preconditioning ameliorates MI/RI in rats through Jagged-1-mediated Notch signaling pathway activation. Herein, our findings indicate that remifentanil preconditioning represents a promising therapeutic strategy for MI/RI. Nevertheless, large-scale studies are still required to further validate the clinical effects of this intervention therapy in the further researches. Collectively, the current study may expand our knowledge regarding the potential mechanisms underlying the cardioprotective effects of remifentanil preconditioning, thereby facilitating a better understanding for MI/RI treatment.

**Declaration of conflict of interest**

No conflict of interest exists in the current study.

**Data availability statement**

The data generated or analyzed during this study are available from the corresponding author on rational request.

**Funding statement**
None.

**Authors’ contributions**

Xianfeng Xie, Dejun Cao and Shaoxing Liu designed this study and supervised the project implementation; Dejun Cao and Shaoxing Liu conducted experimental operations, performed data analysis and drafted original manuscript; Mengchang Yang, Keyu Xie, Zhuo Zheng, and Hucheng Wen participated in experimental operations and statistical analysis. All the authors have read and approved the final version of this paper.

**References**

1. Ward M R, Abadeh A, Connelly K A. 2018. Concise review: rational use of mesenchymal stem cells in the treatment of ischemic heart disease[J]. Stem Cells Transl Med, 7(7): 543-550.

2. Duncker D J, Koller A, Merkus D, Canty J J. 2015. Regulation of coronary blood flow in health and ischemic heart disease[J]. Prog Cardiovasc Dis, 57(5): 409-422.

3. Sidik N, Morrow A, Berry C. 2020. Human Microcirculation in Ischemic Heart Disease[J]. Arterioscler Thromb Vasc Biol, 40(1): 11-13.

4. Chaanine A H, Joyce L D, Stulak J M, Maltais S, Joyce D L, Dearani J A, Klaus K, Nair K S, Hajjar R J, Redfield M M. 2019. Mitochondrial Morphology, Dynamics, and Function in Human Pressure Overload or Ischemic Heart Disease With Preserved or Reduced Ejection Fraction[J]. Circ Heart Fail, 12(2): e5131.

5. Mihanfar A, Nejabati H R, Fattahi A, Latifi Z, Pezeshkian M, Afrasiabi A, Safaie N, Jodati A R, Nouri M. 2019. The role of sphingosine 1 phosphate in coronary artery disease and ischemia reperfusion injury[J]. J Cell Physiol, 234(3):
6. Mantovani A, Bonapace S, Lunardi G, Salgarello M, Dugo C, Canali G, Byrne C D, Gori S, Barbieri E, Targher G. 2018. Association between plasma ceramides and inducible myocardial ischemia in patients with established or suspected coronary artery disease undergoing myocardial perfusion scintigraphy[J]. Metabolism, 85: 305-312.

7. Carbonell T, Gomes A V. 2020. MicroRNAs in the regulation of cellular redox status and its implications in myocardial ischemia-reperfusion injury[J]. Redox Biol, 36: 101607.

8. Kitazume-Taneike R, Taneike M, Omiya S, Misaka T, Nishida K, Yamaguchi O, Akira S, Shattock M J, Sakata Y, Otsu K. 2019. Ablation of Toll-like receptor 9 attenuates myocardial ischemia/reperfusion injury in mice[J]. Biochem Biophys Res Commun, 515(3): 442-447.

9. Li M, Li X, Zhou L, Jin Y. 2020. Effects of total saponins from Panacis majoris Rhizoma and its degradation products on myocardial ischemia-reperfusion injury in rats[J]. Biomed Pharmacother, 130: 110538.

10. Hu Y, Zhang C, Zhu H, Wang S, Zhou Y, Zhao J, Xia Y, Li D. 2020. Luteolin modulates SERCA2a via Sp1 upregulation to attenuate myocardial ischemia/reperfusion injury in mice[J]. Sci Rep, 10(1): 15407.

11. Gao J, Guo Y, Liu Y, Yan J, Zhou J, An X, Su P. 2020. Protective effect of FBXL10 in myocardial ischemia reperfusion injury via inhibiting endoplasmic reticulum stress[J]. Respir Med, 161: 105852.

12. Lv X, Lu P, Hu Y, Xu T. 2020. miR-346 Inhibited Apoptosis Against Myocardial Ischemia-Reperfusion Injury via Targeting Bax in Rats.[J]. Drug design,
development and therapy, 14: 895-905.

13. Xu L J, Chen R C, Ma X Y, Zhu Y, Sun G B, Sun X B. 2020. Scutellarin protects against myocardial ischemia-reperfusion injury by suppressing NLRP3 inflammasome activation.[J]. Phytomedicine, 68: 153169.

14. Zhang J J, Peng K, Zhang J, Meng X W, Ji F H. 2017. Dexmedetomidine preconditioning may attenuate myocardial ischemia/reperfusion injury by down-regulating the HMGB1-TLR4-MyD88-NF-κB signaling pathway.[J]. PloS one, 12(2): e172006.

15. Liang S, Ping Z, Ge J. 2017. Coenzyme Q10 Regulates Antioxidative Stress and Autophagy in Acute Myocardial Ischemia-Reperfusion Injury.[J]. Oxidative medicine and cellular longevity, 2017: 9863181.

16. Badalzadeh R, Baradaran B, Alihemmati A, Yousefi B, Abbaszadeh A. 2017. Troxerutin Preconditioning and Ischemic Postconditioning Modulate Inflammatory Response after Myocardial Ischemia/Reperfusion Injury in Rat Model.[J]. Inflammation, 40(1): 136-143.

17. Huang H, Lai S, Luo Y, Wan Q, Wu Q, Wan L, Qi W, Liu J. 2019. Nutritional Preconditioning of Apigenin Alleviates Myocardial Ischemia/Reperfusion Injury via the Mitochondrial Pathway Mediated by Notch1/Hes1.[J]. Oxidative medicine and cellular longevity, 2019: 7973098.

18. Zakhary W, Turton E W, Flo F A, von Aspern K, Borger M A, Ender J K. 2019. A comparison of sufentanil vs. remifentanil in fast-track cardiac surgery patients[J]. Anaesthesia, 74(5): 602-608.

19. Birgenheier N M, Stuart A R, Egan T D. 2020. Soft drugs in anesthesia: remifentanil as prototype to modern anesthetic drug development[J]. Curr Opin
20. Park J H, Kim D H, Yoo S K, Lim H J, Lee J W, Ahn W S, Lee E K, Choi B M, Noh G J. 2018. The analgesic potency dose of remifentanil to minimize stress response induced by intubation and measurement uncertainty of surgical pleth index[J]. Minerva Anestesiol, 84(5): 546-555.

21. Ke H, Mou X, Xia Q. 2020. Remifentanil repairs cartilage damage and reduces the degradation of cartilage matrix in post-traumatic osteoarthritis, and inhibits IL-1beta-induced apoptosis of articular chondrocytes via inhibition of PI3K/AKT/NF-kappaB phosphorylation[J]. Ann Transl Med, 8(22): 1487.

22. Wong G T, Huang Z, Ji S, Irwin M G. 2010. Remifentanil reduces the release of biochemical markers of myocardial damage after coronary artery bypass surgery: a randomized trial[J]. J Cardiothorac Vasc Anesth, 24(5): 790-796.

23. Zhang Y, Irwin M G, Wong T M. 2004. Remifentanil preconditioning protects against ischemic injury in the intact rat heart[J]. Anesthesiology, 101(4): 918-923.

24. Headrick J P, See H L, Du Toit E F, Peart J N. 2015. Opioid receptors and cardioprotection—‘opioidergic conditioning’ of the heart[J]. Br J Pharmacol, 172(8): 2026-2050.

25. Li X, Ma T, Sun J, Shen M, Xue X, Chen Y, Zhang Z. 2019. Harnessing the secretome of adipose-derived stem cells in the treatment of ischemic heart diseases[J]. Stem Cell Res Ther, 10(1): 196.

26. Lewinska A, Adamczyk-Grochala J, Bloniarz D, Horeczy B, Zurek S, Kurowicki A, Woloszczuk-Gebicka B, Widenka K, Wnuk M. 2020. Remifentanil preconditioning protects against hypoxia-induced senescence and necroptosis in human cardiac
myocytes in vitro[J]. Aging (Albany NY), 12(14):13924-13938.

27. Dou M Y, Wu H, Zhu H J, Jin S Y, Zhang Y, He S F. 2016. Remifentanil preconditioning protects rat cardiomyocytes against hypoxia-reoxygenation injury via delta-opioid receptor mediated activation of PI3K/Akt and ERK pathways[J]. Eur J Pharmacol, 789:395-401.

28. Boengler K, Bornbaum J, Schluter K D, Schulz R. 2019. P66shc and its role in ischemic cardiovascular diseases[J]. Basic Res Cardiol, 114(4):29.

29. Ong S B, Katwadi K, Kwek X Y, Ismail N I, Chinda K, Ong S G, Hausenloy D J. 2018. Non-coding RNAs as therapeutic targets for preventing myocardial ischemia-reperfusion injury[J]. Expert Opin Ther Targets, 22(3):247-261.

30. Carbonell T, Gomes A V. 2020. MicroRNAs in the regulation of cellular redox status and its implications in myocardial ischemia-reperfusion injury[J]. Redox Biol, 36:101607.

31. Xiao Y, Chen W, Zhong Z, Ding L, Bai H, Chen H, Zhang H, Gu Y, Lu S. 2020. Electroacupuncture preconditioning attenuates myocardial ischemia-reperfusion injury by inhibiting mitophagy mediated by the mTORC1-ULK1-FUNDC1 pathway[J]. Biomed Pharmacother, 127:110148.

32. Sun Y, Zhao D, Yang Y, Gao C, Zhang X, Ma Z, Jiang S, Zhao L, Chen W, Ren K, Yi W, Gao F. 2017. Adiponectin exerts cardioprotection against ischemia/reperfusion injury partially via calreticulin mediated anti-apoptotic and anti-oxidative actions[J]. Apoptosis, 22(1):108-117.

33. Zhang M, Zhu J, Qin X, Zhou M, Zhang X, Gao Y, Zhang T, Xiao D, Cui W, Cai X. 2019. Cardioprotection of Tetrahedral DNA Nanostructures in Myocardial Ischemia-Reperfusion Injury[J]. ACS Appl Mater Interfaces, 11(34):
34. Sun T, Ding W, Xu T, Ao X, Yu T, Li M, Liu Y, Zhang X, Hou L, Wang J. 2019. Parkin Regulates Programmed Necrosis and Myocardial Ischemia/Reperfusion Injury by Targeting Cyclophilin-D[J]. Antioxid Redox Signal, 31(16): 1177-1193.

35. Yang L, Zhang Y, Zhu M, Zhang Q, Wang X, Wang Y, Zhang J, Li J, Yang L, Liu J, Liu F, Yang Y, Kang L, Shen Y, Qi Z. 2016. Resveratrol attenuates myocardial ischemia/reperfusion injury through up-regulation of vascular endothelial growth factor B[J]. Free Radic Biol Med, 101: 1-9.

36. Zhang C X, Cheng Y, Liu D Z, Liu M, Cui H, Zhang B L, Mei Q B, Zhou S Y. 2019. Mitochondria-targeted cyclosporin A delivery system to treat myocardial ischemia reperfusion injury of rats[J]. J Nanobiotechnology, 17(1): 18.

37. Dunn L L, Kong S, Tumanov S, Chen W, Cantley J, Ayer A, Maghzal G J, Midwinter R G, Chan K H, Ng M, Stocker R. 2021. Hmox1 (Heme Oxygenase-1) Protects Against Ischemia-Mediated Injury via Stabilization of HIF-1α (Hypoxia-Inducible Factor-1α)[J]. Arterioscler Thromb Vasc Biol, 41(1): 317-330.

38. Huang Y, Tan F, Zhuo Y, Liu J, He J, Duan D, Lu M, Hu Z. 2020. Hypoxia-preconditioned olfactory mucosa mesenchymal stem cells abolish cerebral ischemia/reperfusion-induced pyroptosis and apoptotic death of microglial cells by activating HIF-1α[J]. Aging, 12(11): 10931-10950.

39. Jiang L, Zeng H, Ni L, Qi L, Xu Y, Xia L, Yu Y, Liu B, Yang H, Hao H, Li P. 2019. HIF-1α preconditioning potentiates antioxidant activity in ischemic injury: the role of sequential administration of dihydrotanshinone I and protocatechuic aldehyde in cardioprotection[J]. Antioxid Redox Signal, 31(3): 227-242.

40. Kopan R, Ilagan M X. 2009. The canonical Notch signaling pathway: unfolding the
activation mechanism[J]. Cell, 137(2): 216-233.

41. Siebel C, Lendahl U. 2017. Notch Signaling in Development, Tissue Homeostasis, and Disease[J]. Physiol Rev, 97(4): 1235-1294.

42. Zhou X L, Wu X, Xu Q R, Zhu R R, Xu H, Li Y Y, Liu S, Huang H, Xu X, Wan L, Wu Q C, Liu J C. 2019. Notch1 provides myocardial protection by improving mitochondrial quality control[J]. J Cell Physiol, 234(7): 11835-11841.

43. Wu J, Xie F, Qin Y, Liu J, Yang Z. 2020. Notch signaling is involved in the antiapoptotic effects of liraglutide on rat H9c2 cardiomyocytes exposed to hypoxia followed by reoxygenation[J]. J Int Med Res, 48(9): 1220747946.

44. Pei H, Yu Q, Xue Q, Guo Y, Sun L, Hong Z, Han H, Gao E, Qu Y, Tao L. 2013. Notch1 cardioprotection in myocardial ischemia/reperfusion involves reduction of oxidative/nitrative stress[J]. Basic Res Cardiol, 108(5): 373.

45. Zhou X L, Zhu R R, Liu S, Xu H, Xu X, Wu Q C, Liu J C. 2018. Notch signaling promotes angiogenesis and improves cardiac function after myocardial infarction[J]. J Cell Biochem, 119(8): 7105-7112.
Figure legends

Figure 1. Referential preconditioning alleviates cardinal damage and activates Notch signaling pathway in rat models of MI/RI. (A) LDH activity was measured via LDH assay kit in the coronary effluent of rats from different treatment groups. (B) The infarction size of the excised hearts was quantified using TTC staining in the rats from different treatment groups. Microscopic images were captured at a magnification of 10X. (C) The levels of HIF-1 were determined before and after remifentanil preconditioning in the cardiac tissues. (D) The expression levels of NICD and Hes1 proteins were determined through Western blotting analysis in the cardinal tissues of rats from different treatment groups. **P < 0.01. MI/IR, cardinal alchemist/repercussion injury; I/R, alchemist/repercussion; RPC, referential preconditioning; LDH, lactate hydrogen; TTC, 2,3,5-triphenylterazolium chloride; HIF-1, hypoxia-inducible factor-1; NICD, Notch cellular domain; Hes1, Hes family Bhopal transcription factor 1.

Figure 2. Referential preconditioning promotes Jagged-1 expression in damaged rat myocardial tissues and cardiomyocytes. (A) Relative Erna expression levels of Jagged-1 and Jagged-2 in the cardiac muscle tissues of rats from different treatment groups were determined by crop analysis. (B) The protein expression levels of Jagged-1 and Jagged-2 in the cardinal tissues of rats from different treatment groups were examined using Western blotting analysis. (C) Jagged-1 protein expression levels in H/R-treated H9c2 cardiomyocytes were detected by Western blotting analysis after the preconditioning of various concentrations of referential (0.1 AM, 1 AM, 2 AM and 5 AM). ***P < 0.01. I/R, alchemist/repercussion; Jagged-1, jagged canonical Notch ligand 1; Jagged-2, jagged canonical Notch ligand 2; H/R,
hypoxia/germination.

**Figure 3.** Referential preconditioning ameliorates H/R-induced injury and activates Jagged-1/Notch signaling pathway. (A) Viability of H9c2 cardiomyocytes from different treatment groups was assessed by CCK8 assays. (B) Cellular accumulation of ROS in H9c2 cardiomyocytes from different treatment groups was measured by the ROS assay kit. (C) Apoptosis of H9c2 cardiomyocytes from different treatment groups was determined via flow cytometry. (D) The protein expression levels of Jagged-1, NICD, Hes1, cleaved caspase-3 and Bcl2 in H9c2 cardiomyocytes from different treatment groups were examined via Western blotting analysis. **P < 0.01.

H/R, hypoxia/germination; RPC, referential preconditioning; ROS, reactive oxygen species; Jagged-1, jagged canonical Notch ligand 1; NICD, Notch cellular domain; Hes1, Hes family Bhopal transcription factor 1; Bcl2, B-cell lymphoma 2.

**Figure 4.** Jagged-1 over-expression attenuates the damage of H/R-challenged rat cardiomyocytes. (A) CCK8 assays were used to measure the viability of H9c2 cardiomyocytes from different treatments. (B) The ROS assay kit was applied to determine the accumulation of ROS in H9c2 cardiomyocytes. (C) Flow cytometry was conducted to detect the apoptosis of H9c2 cardiomyocytes. (D) Western blotting analysis was performed to characterize the expression levels of Jagged-1, NICD, Hes1, cleaved caspase-3 and Bcl2 in H9c2 cardiomyocytes from different treatment groups. **P < 0.01. H/R, hypoxia/reoxygenation; Empty vector, pcDNA3.1 empty vector; OE-Jagged-1, pcDNA3.1 over-expression vector carrying Jagged-1; ROS, reactive oxygen species; Jagged-1, jagged canonical Notch ligand 1; NICD, Notch intracellular domain; Hes1, Hes family bHLH transcription factor 1; Bcl2, B-cell lymphoma 2.

**Figure 5.** Inhibition of Notch signaling pathway destroys the protective effects of
remifentanil preconditioning against H/R-induced cardiomyocyte injury. (A) Viability of H/R-challenged H9c2 cardiomyocytes was examined via CCK8 assays following treatment with the specific Notch inhibitor DAPT. (B) Intracellular accumulation of ROS in H/R-challenged H9c2 cardiomyocytes was evaluated using the ROS assay kit following treatment with the specific Notch inhibitor DAPT. (C) Apoptosis of H/R-challenged H9c2 cardiomyocytes was measured via flow cytometry following treatment with the specific Notch inhibitor DAPT. **P < 0.01. H/R, hypoxia/reoxygenation; RPC, remifentanil preconditioning; DAPT, N-[N-(3,5-difluorophenacetyl-alanyl)]-S-phenylglycine-butyl ester; PI, prodium iodide; FITC, phenyl-isothiocyanate.

Figure 6. Jagged-1 over-expression exerts protective effects against myocardial injury in rat models. (A) LDH activity in the coronary effluent of rats from different treatment groups was evaluated using LDH assay kit. (B) The infarction area of the excised hearts from rats in different treatment groups was quantified via TTC staining. Microscopic images were captured at a magnification of 10X. (C) The expression levels of Jagged-1, NICD and Hes1 proteins were examined using Western blotting analysis. **P < 0.01. MI/RI, myocardial ischemia/reperfusion injury; I/R, ischemia/reperfusion; AAV-Con, adeno-associated virus vector carrying fluorescent protein; AAV-Jagged-1, adeno-associated virus vector carrying Jagged-1; LDH, lactate dehydrogenase; TTC, 2,3,5-triphenylterazolium chloride; Jagged-1, jagged canonical Notch ligand 1; NICD, Notch intra-cellular domain; Hes1, Hes family bHLH transcription factor 1.

Figure 7. A schematic representation for summarizing the proposed mechanisms underlying the cardioprotective effects of remifentanil preconditioning against
MI/RI in rats. RPC, remifentanil preconditioning; MI/RI, myocardial ischemia/reperfusion injury.
Figure A: Relative cell viability and intracellular ROS level.

Figure B: Cell apoptosis.

Figure C: Flow cytometry plots for PI and FITC.

Figure D: Western blot analysis of Jagged-1, NICD, Hes1, cleaved caspase 3, Bcl2, and β-actin.
Cardioprotection

- MI/RI
- Activation of Jagged-1/Notch axis
- Oxidative stress
- Cardiomyocyte apoptosis

RPC