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ORIGINAL ARTICLE

κ-Opioid receptors are involved in enhanced cardioprotection by combined fentanyl and limb remote ischemic postconditioning

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Abstract
Background The combination of various interventions to obtain enhanced cardioprotection is always an important area of research focus. This randomized experiment was designed to assess whether combined fentanyl and limb remote ischemic postconditioning produced enhanced protection against myocardial ischemia/reperfusion injury in an in vivo rat model, and to determine if κ-opioid receptors were implicated in the cardioprotection of these interventions.
Methods Seventy-two rats were exposed to a 30-min myocardial ischemia followed by a 180-min reperfusion. Half of the rats (36) were randomized into four different groups receiving control treatment, fentanyl postconditioning, limb remote ischemic postconditioning, and combined fentanyl and limb remote ischemic postconditioning. The remaining 36 rats were also randomized into four groups receiving the same interventions as the above groups following the intravenous administration of a κ-opioid receptor antagonist, nor-binaltorphimine, before myocardial ischemia. At the end of reperfusion, both serum cardiac troponin I and infarct size were determined.
Results Both fentanyl postconditioning and limb remote ischemic postconditioning significantly decreased the infarct size and serum cardiac troponin I level, and combined fentanyl and limb remote ischemic postconditioning produced enhanced cardioprotection on the infarct size-sparing effect. The use of nor-binaltorphimin to block κ-opioid receptors eliminated cardioprotection by fentanyl postconditioning and enhanced cardioprotection by combined fentanyl and limb remote ischemic postconditioning, but did not change cardioprotection by limb remote ischemic postconditioning.
Conclusions Combined fentanyl and limb remote ischemic postconditioning produced enhanced protection against myocardial ischemia/reperfusion injury. κ-Opioid receptors are essential for cardioprotection by fentanyl

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postconditioning and enhanced cardioprotection by combined fentanyl and limb remote ischemic postconditioning; however, they do not play a pivotal role in cardioprotection by limb remote ischemic postconditioning.

Keywords Ischemia reperfusion injury · Cardioprotection · Remote ischemic postconditioning · Pharmacological postconditioning · Opioid receptors

Introduction

Both ischemic preconditioning and postconditioning can provide significant protection against myocardial ischemia/reperfusion injury (IRI); however, they necessitate invasive interventions being applied directly to the ischemic myocardium, which is evidently impractical and even harmful in some clinical settings [1]. Thus, researchers have been seeking drugs that can mimic the benefits of ischemic preconditioning or postconditioning, including volatile and intravenous anesthetics [2], opioid drugs [3], cyclosporine [4], etc. The available literature provides compelling evidence that opioid drugs can confer acute and chronic cardioprotection, regardless of whether they are given before or after myocardial ischemia [3, 5]. Fentanyl is one of the opioid drugs commonly used in clinical anesthesia and its cardioprotection has also been proven in animal trials [6, 7].

Brief episodes of nonlethal ischemia and reperfusion applied to the tissues or organs distal to the heart, known as remote ischemic conditioning, have been shown to reduce myocardial infarct size [8]. Compared with ischemic preconditioning and postconditioning, remote ischemic conditioning is more convenient and safer for patients with acute myocardial infarction because invasive intervention on ischemic myocardium and diseased coronary vessels is not necessary [9]. Of these remote protective interventions, limb remote ischemic conditioning can be employed noninvasively by placing a blood pressure cuff on the upper or lower limb to induce brief ischemia. Furthermore, transient limb ischemia may actually be the best method as it is easy to perform and does not require any invasive intervention. This is an important finding that has greatly eased the translation of ischemic conditioning to clinical practice, especially for limb remote ischemic postconditioning [10]. To date, cardioprotection induced by limb remote ischemic postconditioning has been confirmed in animals [11] and clinical studies [12]. However, compared with ischemic preconditioning, the strength of cardioprotection by limb remote ischemic postconditioning is weaker [13].

It is generally believed that combined different interventions can provide enhanced protection against myocardial IRI [14, 15]. However, no study has evaluated whether pharmacological postconditioning by opioid drugs can enhance cardioprotection conferred by limb remote ischemic postconditioning. Additionally, it has been reported that specific κ-opioid receptors exist on rat cardiac sarcolemma [16], and κ-opioid receptors play an important role in opioid drug preconditioning [17], remote ischemic preconditioning [18], and ischemic postconditioning [19]. It is unclear whether κ-opioid receptors play a similar role in the cardioprotective effects of fentanyl postconditioning, limb remote ischemic postconditioning, and their combination. Thus, this randomized, controlled experiment was conducted to determine if combined fentanyl and limb remote ischemic postconditioning produced enhanced protection against myocardial IRI in an in vivo rat model, and if κ-opioid receptors were implicated in the cardioprotective effects of these interventions.

Methods

Surgical preparation of animals

All animal procedures were approved by the Animal Care and Use Committee of Peking Union Medical College. We used eight- or nine-week-old male Sprague–Dawley rats weighing 290–320 g, provided by Vital River Laboratories (Beijing, China). The rats received humane care in compliance with the 'Guide for the care and use of laboratory animals' established by NIH Publication No. 85-23, revised 1996. Furthermore, all efforts were made to minimize the number of animals used and their suffering during the experiment.

An in vivo rat model with acute myocardial IRI was performed, as previously described [20]. After an intraperitoneal injection of sodium pentobarbital 60 mg/kg, the trachea was intubated and mechanical ventilation was carried out using a rodent respirator with oxygen-enriched room air. By adjusting the ventilation rate and tidal volume, arterial blood gases were maintained at normal limits, with pH of 7.35–7.45, $PaCO_2$ of 30–40 mmHg and PaO_2 of 80–110 mmHg. The body temperature of the rat was maintained at 36.5–37.5 °C using a heating pad. The right internal jugular vein was cannulated to take blood samples for assaying serum cardiac troponin I (cTnI) and for injecting drugs or normal saline. The right carotid artery was cannulated to monitor arterial pressure with a MP150 data acquisition and analysis system (Biopac Systems Inc., CA, USA). The lead II electrocardiogram and heart rate (HR) were continuously recorded by placing subcutaneous needle electrodes on the limbs.

After left thoracotomy through the fourth intercostal space and pericardiotomy, a 5-0 silk ligature was placed under the left coronary artery (LCA), and the ends of the

tie were threaded through a small plastic tube to form a snare for reversible LCA occlusion. Following a 10-min equilibration period, the ligature was tied to block blood flow of the LCA, producing local myocardial ischemia. Successful blockage of LCA was confirmed by the presence of ST-segment elevation on the electrocardiogram and a change in ventricular color from fresh-red to dark-red or paleness. After loosening the LCA ligation, adequate reperfusion of myocardial blood flow was confirmed by visualizing an apparent epicardial hyperemic response and reversion of electrocardiogram changes, and elevated ST descending >50 % of the elevation level [21].

Limb remote ischemic postconditioning

Limb remote ischemic postconditioning was performed by tourniquets around the upper third of the two hind limbs, as previously described [11]. The bilateral hind limbs underwent a 10-min ischemia which started 15 min after LCA ligation, and limb blood supply was restored 5 min before loosening the LCA ligation for myocardial reperfusion. Limb blood flow arrest was confirmed by a change of limb skin color and vascular Doppler. After the tourniquet was released, the limb skin returned to hyperemic color.

Experimental protocol

All animals were exposed to a 30-min myocardial ischemia by LCA ligation and a 180-min myocardial reperfusion by loosening LCA ligation. Seventy-two rats in which the acute myocardial IRI model had been successfully established were randomly allocated to one of eight experimental groups (9 rats in each group) (Fig. 1)—control group (C), fentanyl postconditioning group (F), limb remote ischemic postconditioning group (R), and combined fentanyl and limb remote ischemic postconditioning (F-R), and the other four groups were expressed as n-C, n-F, n-R and n-F-R, respectively. Other than an intravenous injection of 0.5 ml normal saline 15 min after LCA ligation, no additional intervention was executed in group C. In groups F and F-R, fentanyl 30 μg/kg was slowly injected intravenously 15 min after LCA ligation. In groups R and F-R, the rats received the limb remote ischemic postconditioning procedure. The animals in groups n-C, n-F, n-R and n-F-R received the same interventions as the above groups C, F, R and F-R, respectively, after nor-binaltorphimine (nor-BNI), a κ-opioid receptor antagonist was slowly injected intravenously 5 min before LCA ligation [20]. The dosage of nor-BNI was 5 mg/kg and was diluted with 0.5 ml normal saline immediately before use. An independent investigator who did not participate in data recording executed the experimental protocol according to group allocation. The other investigators were blinded to the identity of the treatments used in all groups.

The weight and body temperature of the rats after anesthesia were noted. After a 10-min stabilization period, HR and mean arterial pressure (MAP) were measured and recorded as the baseline values. HR and MAP were also recorded at 1 and 20 min of ischemia, and 60, 120 and 180 min of reperfusion. The rate pressure product (RPP) at every observed time point was calculated as the index of myocardial oxygen consumption [22].

Measurement of serum cTnI

At the end of reperfusion, a 2-ml blood sample was taken from the right internal jugular vein, and serum cTnI was assessed using enzyme-linked immunosorbent assay (ELISA) kits specific for rat factors, following the manufacturer's instructions (RapidBio Co. Ltd, West Hills, CA, USA).

Evaluation of infarct size

After collecting the blood sample at the end of reperfusion, the LCA was again blocked and 1 ml of 2 % Evans blue dye was injected intravenously to distinguish between perfused and nonperfused (area at risk or AAR) sections of the heart. Evans blue dye stains the perfused myocardium, whereas the occluded vascular bed remains uncolored. After the rats were killed with an injection of 10 % potassium chloride, the entire heart was removed, and the right ventricle and right and left atria were cut off using scissors. The left ventricle was cut into approximately five transverse sections from the apex to the ligature, and all tissue slices were rinsed in 0.9 % saline solution at 37 °C for 10 min. The slices were photographed with a digital camera. The slices were then immersed in 1 % solution of 2,3,5-triphenyltetrazolium chloride (TTC) for 15 min at 37 °C. In the presence of intact dehydrogenase enzyme systems (normal myocardium), TTC forms a brick-red color, whereas areas of necrosis lack dehydrogenase activity and therefore do not stain. Thus, the viable areas located within the area at risk were stained red, whereas the necrotic myocardium (within the area at risk) remained unstained (pale yellowish color) if infarcted. After overnight fixation in 10 % formaldehyde, the slices were digitally photographed again. All the photographs were taken with a same digital camera on a fixed framework to make sure that direction and distance from the camera lens to the rat heart slices were identical. An enlarged tracing was then made from each slide and analyzed by planimetry using Adobe Photoshop CS (Adobe Systems Inc., San Jose, CA, USA) by a blinded investigator. The size of the area at risk was related to the size of the left ventricle and was expressed in percent, and the infarct

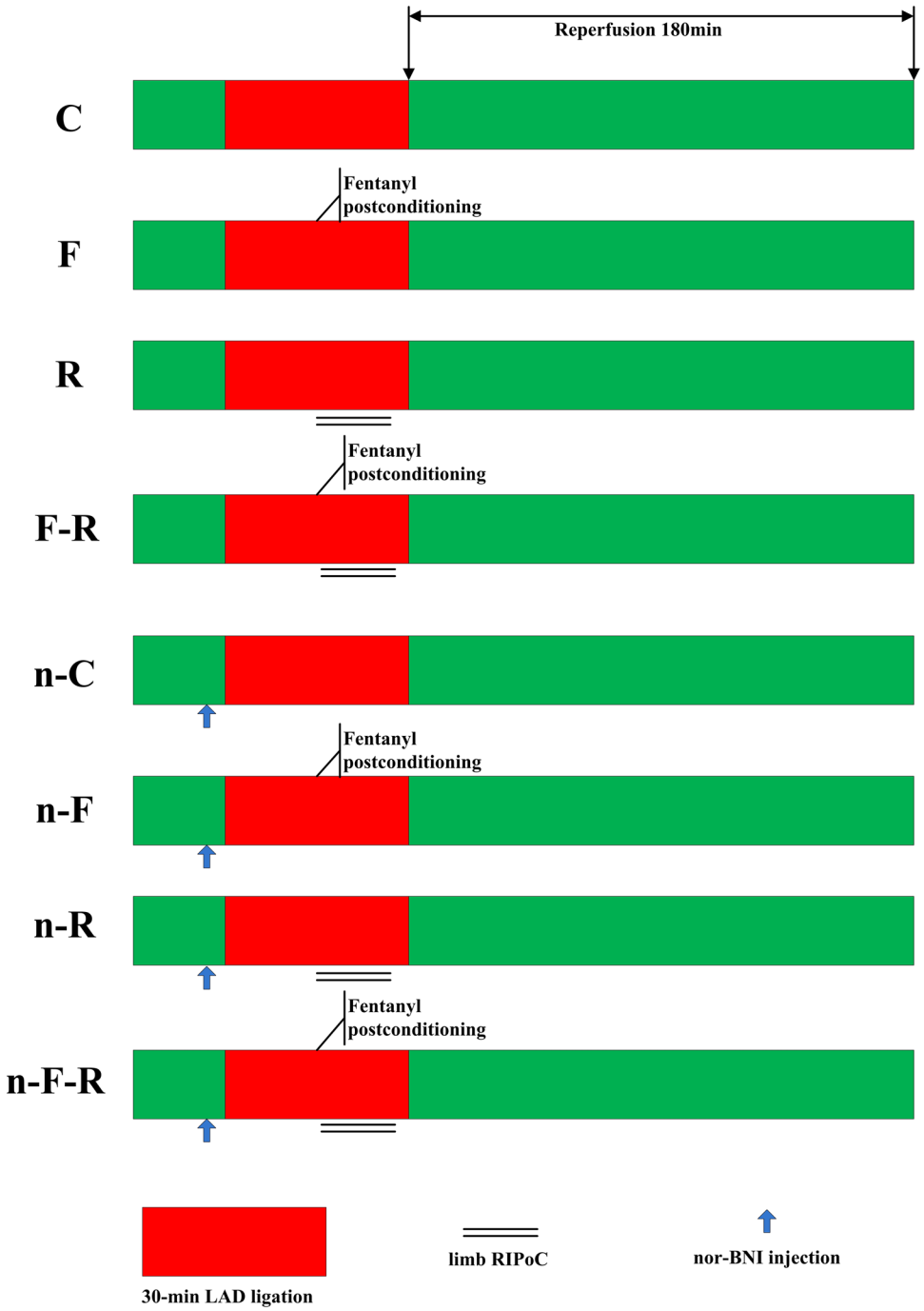

Fig. 1 Experimental protocol. *C* control group, *F* fentanyl postconditioning group, *R* limb remote ischemic postconditioning group, *F-R* combined fentanyl and limb remote ischemic postconditioning group. The animals in groups n-C, n-F, n-R and n-F-R received control treatment, fentanyl postconditioning, limb remote ischemic postconditioning, and combined fentanyl and limb remote ischemic postconditioning, respectively, after intravenous administration of nor-binaltorphimine 5 mg/kg before myocardial ischemia

size related to the size of the area at risk was also expressed in percent [21].

Statistical analysis

Statistical analysis of data was performed with JMP 9 (Version 9.0.2, SAS Institute Inc., Cary, NC, USA). The Kolmogorov–Smirnov test was used to test the normality of the distribution for the parametric data from all study groups. Furthermore, the Levene median test was adopted to test homogeneity of variance for the parametric data. If the data were normally distributed and had homogeneous variance, they were expressed as mean ± SD, and were compared by analysis of variance followed by the Student–Newman–Keuls test. Repeated-measures analysis of variance was applied for intra-group comparisons. When data were not normally distributed or had inhomogeneous variance, they were expressed as median (interquartile range) and compared using the Kruskal–Wallis test and Mann–Whitney *U* test.

Table 1 Hemodynamic changes associated with the myocardial ischemia–reperfusion process

Groups	Baseline	Ischemia			Reperfusion			
		1 min	15 min	20 min	5 min	60 min	120 min	180 min
HR (bpm)								
C	371 ± 39	405 ± 43†	364 ± 54	368 ± 47	373 ± 50	347 ± 34	350 ± 38	349 ± 40
F	398 ± 24	429 ± 19†	399 ± 59	357 ± 29$^{\#,\dagger}$	380 ± 36	398 ± 32	384 ± 36	389 ± 29
F-R	386 ± 20	419 ± 18†	400 ± 32	348 ± 27$^{\#}$	371 ± 42	381 ± 21	386 ± 28	389 ± 21
R	363 ± 33	402 ± 27†	360 ± 47	371 ± 40	386 ± 38	384 ± 39	386 ± 47	375 ± 54
n-C	394 ± 12	433 ± 31†	413 ± 15	396 ± 36	395 ± 31	412 ± 22	380 ± 29	367 ± 36
n-F	394 ± 14	418 ± 13†	397 ± 15	364 ± 19$^{\dagger,\#}$	394 ± 29	390 ± 22	384 ± 9	38 ± 14
n-R	393 ± 13	421 ± 9†	393 ± 13	357 ± 18$^{\dagger,\#}$	375 ± 32	399 ± 11	402 ± 9	390 ± 11
n-F-R	388 ± 11	411 ± 13†	399 ± 22	358 ± 10	396 ± 8	394 ± 11	382 ± 7	382 ± 10
MAP								
C	108 ± 9	76 ± 7†	95 ± 19	95 ± 17†	86 ± 15†	83 ± 17†	77 ± 17†	71 ± 20†
F	111 ± 12	87 ± 17†	101 ± 16	83 ± 6$^{\#,\dagger}$	84 ± 12†	96 ± 9	88 ± 15†	90 ± 9$^{\#,\dagger}$
F-R	114 ± 4	87 ± 7†	108 ± 9	83 ± 8$^{\#,\dagger}$	96 ± 14	102 ± 10†	97 ± 8†	91 ± 9$^{\#,\dagger}$
R	112 ± 8	84 ± 12†	102 ± 13	97 ± 14	90 ± 18†	91 ± 19†	82 ± 12†	82 ± 13$^{\#,\dagger}$
n-C	100 ± 11	78 ± 6†	100 ± 13	99 ± 16	81 ± 23	78 ± 23	72 ± 15	72 ± 11
n-F	102 ± 7	80 ± 12†	99 ± 9	75 ± 22$^{\dagger,\#}$	77 ± 17	94.4 ± 12	85 ± 10	82 ± 7
n-R	99 ± 11	73 ± 11†	91 ± 11	79 ± 10$^{\dagger,\#}$	77 ± 16	91.8 ± 12	90 ± 12	90 ± 12
n-F-R	105 ± 6	79 ± 10†	96 ± 12	92 ± 9	93 ± 10$^{\#}$	86 ± 6	83 ± 6	78 ± 7
RPP (1/1000)								
C	42 ± 4	32 ± 3†	36 ± 8	36 ± 7	34 ± 8†	33 ± 6†	34 ± 9†	30 ± 7†
F	43 ± 6	37 ± 9†	40 ± 10	29 ± 4$^{\#,\dagger}$	31 ± 5†	38 ± 6†	33 ± 7†	35 ± 7$^{\#,\dagger}$
F-R	47 ± 2	38 ± 5	50 ± 6	34 ± 4$^{\#}$	41 ± 7†	45 ± 4	44 ± 5$^{\#,\dagger}$	42 ± 3$^{\#,\dagger}$
R	47 ± 4	39 ± 5	41 ± 8	41 ± 7†	40 ± 9	41 ± 9	39 ± 7†	37 ± 6$^{\#,\dagger}$
n-C	46 ± 3	40 ± 2†	45 ± 5	44 ± 5$^{\#}$	39 ± 10†	40 ± 11	36 ± 7†	34 ± 5†
n-F	46 ± 4	39 ± 6†	44 ± 4	32 ± 9$^{\dagger,\#}$	35 ± 8†	42 ± 6	38 ± 4†	35 ± 3†
n-R	44 ± 4	36 ± 4†	40 ± 4	32 ± 4$^{\dagger,\#}$	33 ± 7†	42 ± 6	40 ± 5	39 ± 5†
n-F-R	47 ± 3	38 ± 4†	42 ± 6	37 ± 4†	42 ± 4	40 ± 3†	37 ± 2†	35 ± 1†

The animals in groups n-C, n-F, n-R and n-F-R received control treatment, fentanyl postconditioning, limb remote ischemic postconditioning, and combined fentanyl and limb remote ischemic postconditioning, respectively, after intravenous administration of nor-binaltorphimine 5 mg/kg before myocardial ischemia

Data are expressed as mean ± SD

HR heart rate, *MAP* mean arterial pressure, *RPP* rate pressure product, *bpm* beats/min, *C* control group, *F* fentanyl postconditioning group, *R* limb remote ischemic postconditioning group, *F-R* combined fentanyl and limb remote ischemic postconditioning group

† $P < 0.05$ compared with baselines; $^{\#}$ $P < 0.05$ compared with group C

A P value of <0.05 was considered statistically significant for all tests.

The hypothesis of this study was that there would be a 20 % difference between the control and treatment groups in infarct size and serum cTnI levels. To detect this difference with a power of 80 % and a P value of 0.05, power calculation indicated that a sample size of at least seven rats per group would be required. More than seven rats per group were included in the study so as to ensure sufficient data to fit the analysis of variance models and to allow for comparisons among other variables of interest.

Results

Basic data and hemodynamics

The body weight, body temperature after anesthesia, and baselines of HR, MAP, and RPP did not differ among the eight groups. Hemodynamic changes during the ischemia and reperfusion process in the eight groups are shown in Table 1. Compared to baselines, at 1 min of ischemia, HR in all eight groups increased significantly, but MAP and RPP decreased significantly. At 3–5 min of ischemia, HR,

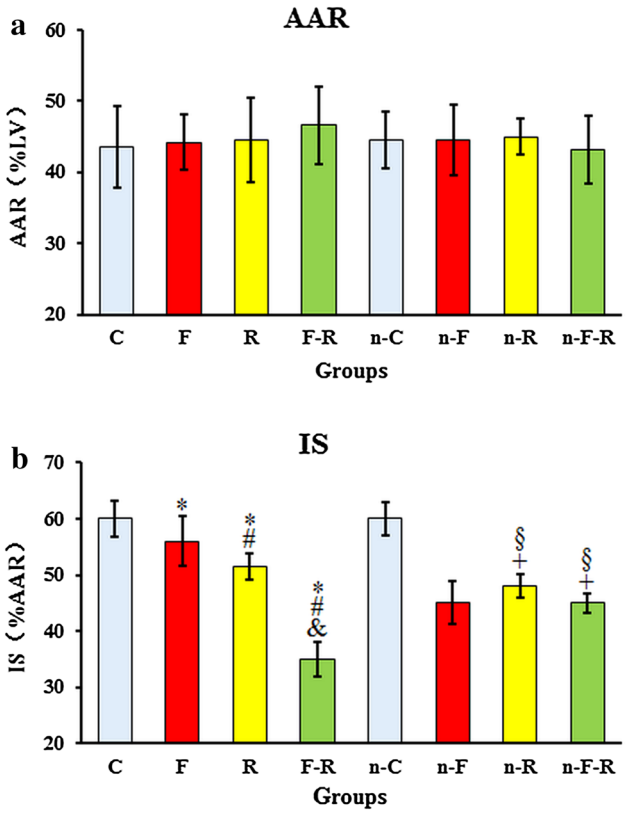

Fig. 2 **a** The areas at risk (AAR) (nonperfused myocardium) of the eight groups. The AAR is expressed as a percentage of the left ventricle. **b** The infarct size (IS) of the eight groups. IS is expressed as a percentage of the AAR ($n = 9$ in each group). Each column shows the average size of AAR or IS of each group. The *short line* is the standard deviation in each group. $^{*}P < 0.05$ compared with group C; $^{\#}P < 0.05$ compared with group F; $^{\&}P < 0.05$ compared with group R; $^{\S}P < 0.05$ compared with group n-C; $^{+}P < 0.05$ compared with group n-F

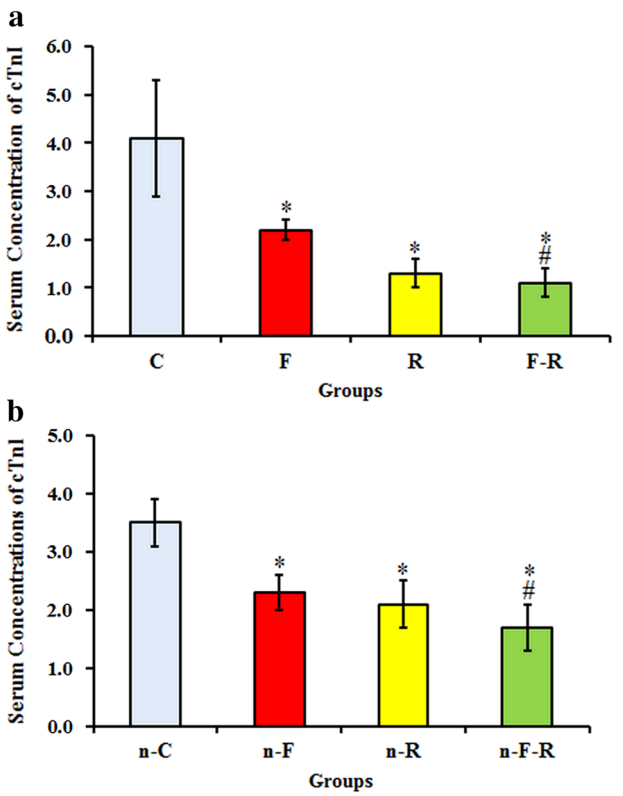

Fig. 3 **a** Serum cTnI levels in groups C, F, R and F-R. **b** Serum cTnI levels in groups n-C, n-F, n-R and n-F-R. Data are expressed as mean ± SD ($n = 9$ in each group). $^{*}P < 0.05$ compared with group C; $^{\#}P < 0.05$ compared with group F

MAP, and RPP returned to baselines in most rats, and there were no differences at 15 min among the eight groups.

After 20 min of ischemia, MAP and RPP decreased significantly in groups F, F-R, n-F and n-F-R compared with groups C, R, n-C and n-R, HR. During reperfusion, HR did not significantly differ among the eight groups, but MAP and RPP decreased gradually with reperfusion in the eight groups.

Areas at risk and infarct sizes

The areas at risk are shown in Fig. 2a. There were no significant differences in the areas at risk among the eight groups. Infarct sizes of all rats are shown in Fig. 2b. Compared with group C (60 ± 3 %), infarct size was significantly smaller in groups F (56 ± 2 %), R (48 ± 1 %) and F-R (35 ± 2 %), but it was not substantially different in group n-C (60 ± 3 %). Infarct size was significantly smaller in group F-R than in groups F and R.

Compared with group n-C, infarct size was not significantly different in group n-F (58 ± 4 %), but it was significantly reduced in groups n-R (45 ± 4 %) and n-F-R (45 ± 2 %). Infarct size was not significantly different between groups R (48 ± 1 %), n-R (45 ± 4 %) and n-F-R (45 ± 2 %).

Serum cTnI level

Serum cTnI level was 4.07 ± 1.21, 2.24 ± 0.19, 1.29 ± 0.29, and 1.09 ± 0.33 ng/ml in groups C, F, R and F-R, respectively (Fig. 3a). Serum cTnI level was significantly decreased in groups F, R and F-R compared with group C. Serum cTnI level was significantly lower in groups R and F-R than in group F, but it was not significantly different between groups R and F-R.

Serum cTnI levels in groups n-C, n-F, n-R and n-F-R were 3.49 ± 0.39, 2.29 ± 0.29, 2.12 ± 0.36 and 1.69 ± 0.39 ng/ml, respectively (Fig. 3b). Serum cTnI level was not significantly different between groups C and n-C.

Compared with group n-C, serum cTnI level was significantly reduced in groups n-F, n-R, and n-F-R. Serum cTnI level was significantly lower in group n-R than in group n-F, but it did not differ between groups n-R and n-F-R.

Discussion

This is the first animal experiment comparing the cardioprotective effects of fentanyl postconditioning, limb remote ischemic postconditioning and their combination in an in vivo rat model of acute myocardial IRI. Considering that use of a high dose of fentanyl during myocardial ischemia can result in significantly adverse hemodynamic effects in the rat [6], a relevantly small dose of fentanyl was chosen for this study. Our results showed that hemodynamic changes induced by intravenous fentanyl during myocardial ischemia were transient and acceptable. Moreover, hemodynamic variables during reperfusion were not significantly different between the groups with and without fentanyl, which is in accordance with the findings of Kato et al. [23] in an in vitro rat heart model. Additionally, homogenization of hemodynamic changes during ischemia and reperfusion in the eight experiment groups also is an important prerequisite to ensure accurate comparisons of myocardial injury results between the groups. Our study showed that pharmacological postconditioning using a small dose of fentanyl produced a 7 % infarct size-sparing effect, i.e., weak cardioprotection.

In our study, limb remote ischemic postconditioning produced a 20 % infarct size-sparing effect, which is significantly weaker than cardioprotection of ischemic preconditioning reported in a study by Sandhu et al. [24] and cardioprotection of ischemic postconditioning reported in a study by Zatta et al. [20]. This may be because compared with the stuttering ischemic stimulus on reperfused myocardium by ischemic preconditioning or postconditioning, limb remote ischemic postconditioning by placing a blood pressure cuff on the lower limb in our study only triggers a single weaker stimulus. Ren et al. [25] demonstrated that the protective effect of limb remote ischemic postconditioning depended on the number and duration of the limb ischemic stimulus, i.e., more potent cardioprotection would be obtained if the circle of limb ischemia is increased or the duration of limb ischemia is prolonged, as performed in a study by Schmidt et al. [13] in a pig model. However, from a practical profile, it is impossible to keep the limbs of an emergency patient in the circles of ischemia and perfusion to acquire more cardioprotection before arriving at the hospital. Consequently, combined use of additional maneuvers that can enhance cardioprotection of limb remote ischemic postconditioning may be a better strategy, as previous studies have proved that combined different interventions can provide synergistic cardioprotection [14, 15, 26].

Our results showed that combined fentanyl and limb remote ischemic postconditioning achieved a 40 % infarct size-sparing effect, which was significantly larger than the total cardioprotection (27 %) from the use of the two treatments taken alone. This suggests that enhanced cardioprotection is obtained by combined fentanyl and limb remote ischemic postconditioning. According to these preliminary findings, we believe that the combined use of simple and weak stimuli such as fentanyl and limb remote ischemic postconditioning, which are obviously simpler and safer in the clinical setting, can produce significant enhanced protection against myocardial IRI. This may provide a useful clue as to how to find an optimal protocol with significant cardioprotection using simple, clinical strategies.

It has been shown that cardioprotection by morphine is mediated by κ-opioid receptors in an in vivo rat model of myocardial IRI [27]. Furthermore, Peart et al. [28] validated that activation of κ-opioid receptors at the beginning of reperfusion provided significant protection against myocardial IRI in both the rat and mice heart models. Obviously, κ-opioid receptors are implicated in cardioprotection before and after myocardial ischemia. Our study showed that with 'beforehand' use of nor-BNI to block κ-opioid receptors, the infarct size-sparing effect of fentanyl postconditioning disappeared, and combined fentanyl postconditioning and limb remote ischemic postconditioning did not produce an enhanced cardioprotective effect, whereas cardioprotection of limb remote ischemic postconditioning did not change. These results suggest that κ-opioid receptors play a pivotal role in the cardioprotective mechanisms of fentanyl postconditioning and in the mechanisms of enhanced cardioprotection by combined fentanyl postconditioning and limb remote ischemic postconditioning, but are not involved in the cardioprotective mechanism of limb remote ischemic postconditioning.

There are some design limitations to our experiment that deserve special attention. First, our study used a classic in vivo model of myocardial IRI; however, an in vivo model cannot tell us whether the cardioprotection of fentanyl postconditioning is initiated by the direct action of the drug on the myocardium or somewhere else (such as reducing cardiovascular stress, etc.). In an in vitro model of myocardial IRI, adding fentanyl or/and opioid receptor antagonists into the perfusion solution may obtain more detailed information about the cardioprotective mechanism of this treatment. Second, the strength of the cardioprotection produced by limb remote ischemic postconditioning is related to tissue volume exposed to the protocol of ischemic stimulus, duration of ischemia, and numbers of circles [29]. In our study design, only cardioprotection of combined limb remote ischemic postconditioning with a single protocol was assessed. We have reasons to suspect that different results are likely to be obtained if various

protocols of limb remote ischemic postconditioning are used. Third, this study did not observe the cardioprotective effects of combinations of different doses of fentanyl and limb remote ischemic postconditioning protocols. Thus, we cannot obtain a dose–response curve by isobolographic analysis to determine whether interaction between fentanyl and limb remote ischemic postconditioning has an additive or synergetic effect [30]. Further studies addressing the above issues are necessary.

In conclusion, in an in vivo rat heart model, remote ischemic postconditioning induced by brief limb ischemia provides more potent protection against myocardial IRI than pharmacological postconditioning induced by a small dose of fentanyl. Moreover, combined fentanyl and limb remote ischemic postconditioning produces enhanced cardioprotection. κ-Opioid receptors are implicated in cardioprotective mechanisms of fentanyl postconditioning and of combined fentanyl and limb remote ischemic postconditioning; however, they do not play a pivotal role in cardioprotection by limb remote ischemic postconditioning.

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Conflict of interest The authors declare that they have no competing interests.

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