# Local anaesthetic ropivacaine protects rats from myocardial ischaemia/reperfusion injury by inhibition of COX-2

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## Abstract

**Introduction:** Myocardial ischaemia/reperfusion (I/R) injury is the leading cause of morbidity and mortality worldwide. Despite novel advances in therapeutics, the management of myocardial I/R is still an unmet medical need. Therefore, in the present study, we have demonstrated the protective effect of ropivacaine (RPC) on the myocardial infarction in rats and its underlying mechanism.

**Material and methods:** Initially, the effect of RPC was determined on the infarct size and histopathology of cardiac tissues. The effect of RPC was also determined on the levels of various cardiac biomarkers such as creatine kinase (CK), creatine kinase MB (CK-MB), alanine aminotransferase (ALT), asparganine aminotransferase (AST), and lactate dehydrogenase (LDH), and biomarkers of oxidative stress (MDA, SOD, and GSH) and inflammation (tumour necrosis factor-α (TNF-α), interleukin 1β (IL-1β), and IL-6). RPC effect was also quantified on cellular apoptosis and COX-2 and iNOS expression via western blot analysis. The RPC was further docked into the active site of COX-2.

**Results:** It has been found that RPC reduces the improves haemodynamics of (LVSP and ± $dp/dt_{max}$, and LVEDP), infarct percentage and architecture of cardiac tissues of rats. It also reduces the level of studies cardiac injury biomarkers together with a reduction of oxidative stress (MDA, SOD, and GSH) and inflammation (TNF-α, IL-1β, and IL-6). Upon administration of RPC, the rate of cellular apoptosis was found to be greatly reduced, with a reduction in COX-2 and iNOS expression. In docking analysis, RPC creates van der Waals forces and pi-interactions with Tyr381, Arg106, Val102, Leu345, Val509, Ser339, Leu338, Val335, Ala513, His75, and Leu517 at the catalytic site of COX-2.

**Conclusions:** Collectively, our results demonstrated that ropivacaine showed significant benefit against myocardial ischaemic injury.

**Key words:** ropivacaine, oxidative stress, inflammation, apoptosis, docking, COX-2.

## Introduction

Despite various advances in diagnostics and therapeutics, myocardial infarction is still posing a significant threat to mankind. It remains a leading cause of morbidity and mortality throughout the world [1]. Dramatic

changes in lifestyle and eating habits predispose a significant number of individuals to myocardial infarction across the globe in the coming years [2, 3]. Particularly in China, as per the Chinese disease report published in 2020, cardiovascular diseases account for approximately 45% of deaths in China [4, 5]. The loss of blood supply to the heart is the main factor for myocardial infarction, which leads to ischaemia of the cardiomyocytes and subsequent necrosis of cardiac tissue [6]. Thus, the majority of drugs used against myocardial ischaemia have been aimed to restore the lost blood supply to prevent infarction, by a combination of agents, which include vasopressor agents, calcium channel antagonists, antiarrhythmic agents, antihypertensive agents, angiotensin convertase enzyme (ACE) inhibitors, and vasodilator. This signifies that none of the single agents can be used to control myocardial infarction and associated ischaemia. Moreover, the restoration of blood supply is not the only factor that can improve the prognosis of patients, but subsequent reperfusion also worsens the situation [7, 8]. This causes the injury to the myocardial tissues known as myocardial ischaemia/reperfusion injury. The generation of reactive oxygen species (ROS) in the reperfusion stage promotes oxidative stress due to the disparity between antioxidant defence mechanisms. It also promotes lipid peroxidation, tissue infiltration, alteration in vascular permeability, and production of various inflammatory cytokines that lead to inflammation [9–11]. Therefore, studies have proven the effectiveness of antioxidants and anti-inflammatory agents in MI/R injury [12–14].

Studies have shown the importance of local anaesthetics (LA) as antiarrhythmic agents. Some clinically relevant LAs are established antiarrhythmic agents, such as lignocaine, which provides an antiarrhythmic effect via inhibition of cardiac sodium channel [15], procainamide impairs the myocardial contractile force and lowers cardiac output and systemic arterial pressure to provide antiarrhythmic effects [16], and prilocaine prevents aconitine-induced arrhythmias [17]. Another LA, bupivacaine, in isolated cardiac tissues, decreases intra-cardiac conduction velocity and contractile force and depresses spontaneous sinoatrial activity. It also decreases cardiac output, myocardial contractility, and intra-cardiac conduction velocity, as shown by increased PR and QRS durations in anaesthetized animals [18]. However, it was later found to possess significant cardiotoxicity, which limits its use against myocardial diseases [19, 20]. Thus, it could be suggested that the antiarrhythmic action of LA is similar to local anaesthesia, in that they prevent the generation of action potential mainly by blocking voltage-gated $Na^+$ channels, $Ca^{2+}$ channels, and $K^+$ channels, which results in membrane stability [21, 22]. Ropivacaine (RPC) is an amide-based non-cardiotoxic local anaesthetic used during surgery, labour, and post-operative pain in adults and children [23, 24]. It blocks a signalling cascade related to tumour necrosis factor $\alpha$ (TNF-$\alpha$), which results in the protection of endothelium [25]. It also protects experimental acute lung injury induced by bacterial lipopolysaccharide (LPS) via reduction of inflammation [26, 27]. The excellent anticancer activity of RPC was identified against cervical, gastric, and hepatic cancer cells [28–31]. Prompted by the above, the present study intended to investigate the effect of RPC on myocardial infarction in rats and its underlying mechanism.

## Material and methods

### Animals

Adult male Sprague-Dawley (8–10 weeks, 240–270 g) rats were obtained from the institutional animal house and kept in a strict hygienic and controlled laboratory environment with an *ad libitum* supply of food and water. The experiment was duly approved by the institutional ethical committee of The First People's Hospital of Fuyang Hangzhou and was performed according to the national guidelines of animal care and use of China.

### Initiation of experimental myocardial ischaemia/reperfusion (I/R) injury

To establish myocardial I/R injury, the rats were fasted for 12 h before and had free access to water. The rats showing abnormal ECG were excluded from the experiment, and only normal rats were selected for further experiments. The selected rats were anaesthetized with 1.5 ml/kg sodium pentobarbital (30 g/l) by i.p. subjected to the tracheal cannula. The breathing frequency of rats was maintained at 50–60 beats per minute using an ALC-V model animal ventilator with continuous monitoring by ECG. A 2-cm longitudinal incision was made on the chest of rats, where the sternum muscle was fixed with a haemostatic clamp. A small orifice was created at the intercostal region of the $2^{nd}$–$3^{rd}$ ribs in the left side near the sternum. The heart of the rat was exposed after removing the pericardium, and a needle and thread were inserted at the lower end of the left atrial appendage to ligate the left anterior descending artery (LAD). The increase in ST-segment showed the successful creation of the MI model. The successful ligation showed reduced blood pressure and prominent ECG changes in rats. After reperfusion, local red colour was shown, and depression of ST-segment was observed on ECG. The chest of the control group rats was opened

and threaded without ligation. In the I/R group the rats were subjected to ligation of the left ventricle for 30 min, and then reperfusion for 120 min. In the treatment group, RPC was administered 30 min before the ischaemia followed by a 5-min interval, and ligation was performed for 30 min with subsequent reperfusion for 120 min.

### Experimental design

Thirty SD rats were taken and randomly divided into 5 groups containing 6 animals in each group.

– group 1: normal control;
– group 2: IR rats;
– group 3: IR + RPC (10 mg/kg);
– group 4: IR + RPC (15 mg/kg);
– group 5: IR + RPC (20 mg/kg).

Different doses of RPC as indicated above were administered immediately through intraperitoneal injection after being dissolved in 0.9% NaCl including 1% DMSO 30 min before the myocardial ischaemia models were completed. All rats then received their respective treatments once daily until the end of the experiment. Thereafter, the rats were killed under anaesthetized conditions using urethane (1 g/kg, intra-peritoneally injected) to isolate blood. The heart tissues were washed in ice-cold normal saline after harvesting.

### Assessment of haemodynamic and cardiac function

The left ventricular systolic pressure (LVSP) and left ventricular end-diastolic pressure (LVEDP) were recorded at the start of the experiment, before ischaemia, after 30-min ischaemia, and after 120-min reperfusion. Values of $+dp/dt_{max}$ and $-dp/dt_{max}$ were calculated using the analysing system of a RM6240 multichannel physiological signal detector.

### Determination of infarct size

To determine myocardial infarct area the Even's blue-2,3,5-triphenyl tetrazolium chloride (TTC) method was used. Briefly, the Evans blue was administered into the tail vein, and non-ischaemic myocardial tissues was coloured dark blue. At this stage, the hearts were excised from the rats and weighed after drying on filter paper. The LV was excised from the heart, weighed, frozen in for 1 h at –20°C, and then sliced into 6 slices along the axis. These slices were then placed into 1% TTC at pH 7.4 and incubated at 37°C followed by soaking in 10% formaldehyde for 15 min. The slices were then photographed, and their weight was recorded.

The infarct size was observed as a grey-white colour due to the absence of dehydrogenase in dead cells, which is unable to stain because dead cells cannot reduce TTC to a deep red colour. The myocardial ischaemic area was measured as per the earlier reported procedure [32].

### Determination of serum LDH and CK levels

The serum concentration of myocardial enzymes, lactate dehydrogenase (LDH), and creatine kinase myocardial band (CK) was recorded using marketable assay kits as per the instructions provided, using a microplate reader at 340 nm. (Nanjing Jiancheng Bioengineering Institute, China).

### Evaluation of lipid peroxidation and antioxidant enzyme levels

The MDA level, SOD, and GSH activities in the heart homogenate supernatant was determined as per the supplier's instructions, using a microplate reader at 560 and 532 nm (Nanjing Jiancheng Bioengineering Institute, China).

### Determination of TNF-α, IL-1β, and IL-6 levels in cardiac tissues

The serum level of tumour necrosis factor-α (TNF-α) and interleukin (IL) 6 was determined by commercial ELISA kits as per the supplier's instructions, using a microplate reader at 450 nm (Nanjing Jiancheng Bioengineering Institute, China).

### Myocardial cell apoptosis

Briefly, the cells were lysed with trypsin and washed twice with PBS. 20 µl Annexin-V-FITC labelling solution was added to 1 ml buffer solution, and then 20 µl PI reagent was added to the resulting cell after lysing. The cells were then kept at room temperature without light for 5 min. The apoptosis was measured by flow cytometer FACScan flow cytometer (Becton Dickinson Company, USA) using CellQuest software (ver 4.0; BD Biosciences).

### Western blot analysis

The isolated protein was loaded on SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and shifted onto the (polyvinylidene difluoride) PVDF membrane and probed with primary antibody. Following incubation with HRP-conjugated goat anti-rabbit IgG for 1 h at room temperature, the protein bands were analysed using an enhanced chemiluminescence reagent by using ImageJ v1.42q software (National Institutes of Health, Bethesda, MA, USA).

### Docking study

The docking study was conducted using ropivacaine as the ligand into the 3D crystal structure of the COX-2 protein model. The CDOCKER

protocol of Discovery Studio (3.0) was selected to perform this study. The protein preparation, ligand preparation, active site identification, and docking were performed using the default setting of the CDOCKER protocol as per the manufacturer's instruction. The receptor-ligand interaction of top-ranked posed of ropivacaine was visualized using suitable scripts of the software. Moreover, the 2D interaction diagram was constructed using 2D interaction viewer commands.

### Ethical approval

The study was approved by the First People's Hospital of Fuyang Hangzhou. All experiments were conducted under internationally accepted principles for laboratory animal use and care, as found in the United States guidelines (NIH publication no. 85–23, revised in 328 1985).

### Statistical analysis

Data were analysed by SPSS 17.0 software and expressed as the means ± SD. Differences were analysed by one-way analysis of variance (ANOVA) followed by Dunnett's test for individual comparisons between each group mean. A *p*-value of 0.05 was considered statistically significant.

## Results

### The effect of RPC on the haemodynamic parameters

The effect of RPC was first investigated on the haemodynamic parameters in anaesthetized rats. The results are shown in Figure 1. After myocardial I/R injury, the disease control rats showed a decreased level of LVSP and $\pm dp/dt_{max}$, and LVEDP was found to be significantly increased as compared to the sham. These effects were partly restored to near normal after treatment with RPC in a concentration-dependent manner. These results were further substantiated with ECG analysis of rats following myocardial ischaemia-reperfusion injury (supplementary Figure S1). It was found that pre-treatment of RPC caused a significant reduction in the ST-segment of rats as compared to IR rats.

### The effect of RPC on the myocardial infarct area

As shown in Figure 2 A, the infarct size was found to greatly increased in the IR group with no treatment as compared to the sham. The RPC-treated group showed a significant reduction in the infarct size of the animals in a dose-de-

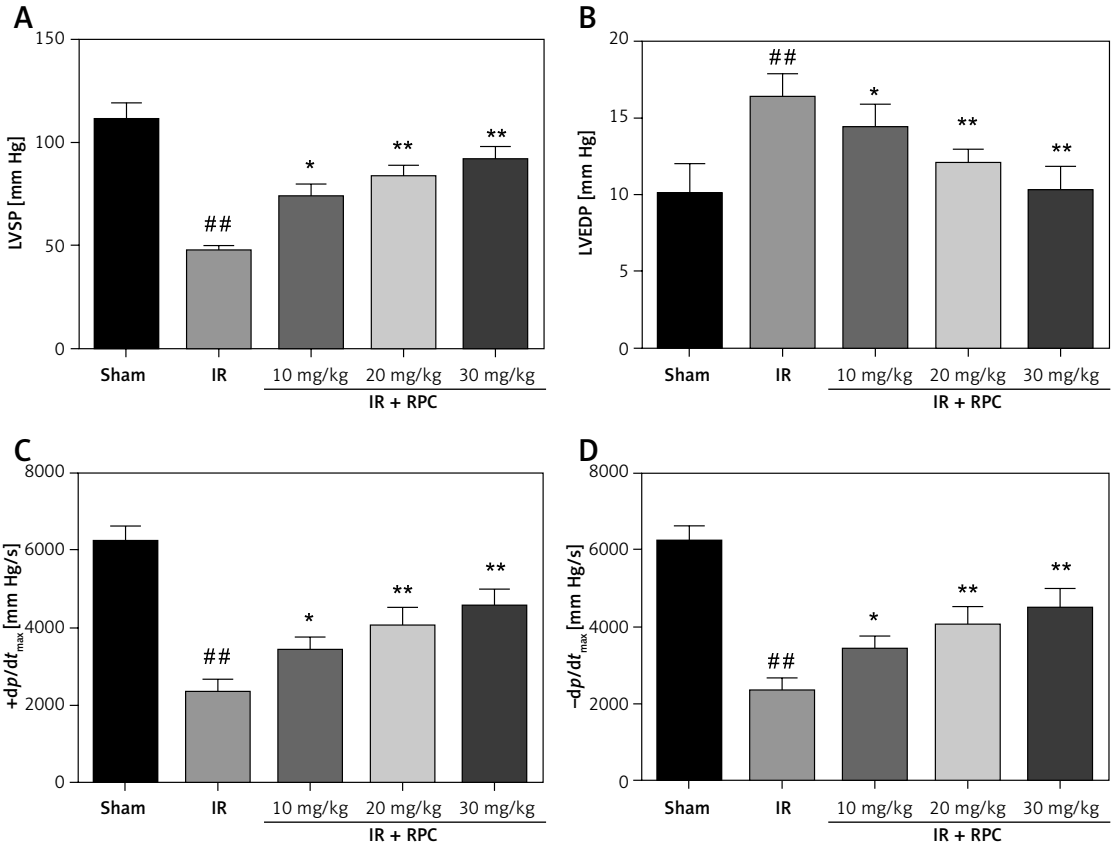


**Figure 1.** Effect of RPC on the haemodynamic parameter after MI/R in rats. **A** – LVSP, **B** – LVEDP, **C** – $+dp/dt_{max}$, **D** – $-dp/dt_{max}$. Data expressed as means ± SDs. $^{\#\#}p < 0.05$ compared with sham group; $^{*}p < 0.05$, $^{**}p < 0.01$ compared with I/R group

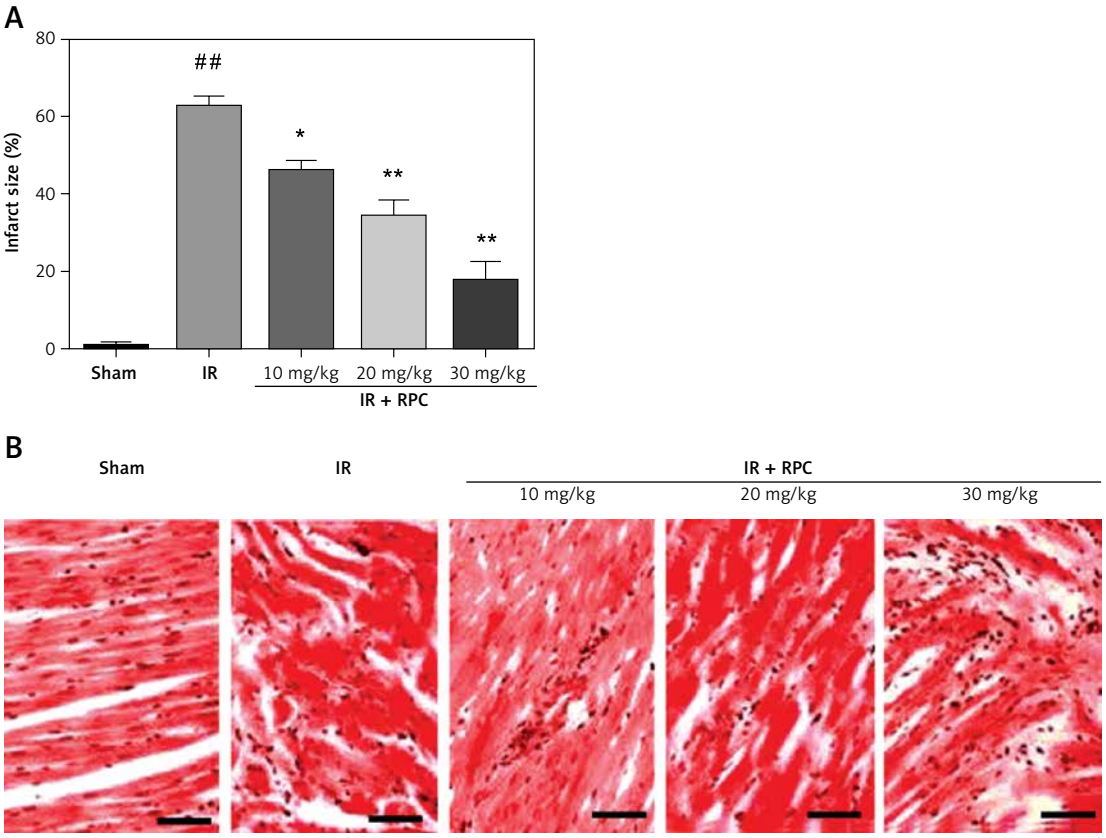


**Figure 2.** Effect of RPC on the (**A**) infarct volume and (**B**) histopathology of myocardial tissue by H and E analysis. Data were expressed as means ± SD. $^{\#\#}p < 0.05$ compared with sham group; $^{*}p < 0.05$, $^{**}p < 0.01$ compared with I/R group

pendent manner in comparison to the IR control group.

### The effect of RPC on the histopathology of cardiac tissues

As shown in Figure 2 B, the IR group showed marked necrosis and inflammation of myocardial tissues, which was absent in the sham-treated group. However, upon administration of RPC, these histopathological changes were returned near to normal, as confirmed by reduced oedema, necrosis, and inflammation in a dose-dependent manner. This observation confirmed that RPC would be able to mitigate the after-effects of MRI in rats.

### The effect of RPC on the cardiac injury biomarkers

The effect of RPC was further evaluated on various biomarkers of cardiac injury, and the results are presented in Figure 3. It was found that the concentrations of various tested biochemical mediators (e.g. CK, CK-MB, ALT, AST, and LDH) were highly elevated in the IR group, which directly correlates with the myocardial injury as compared to the sham. The RPC-treated group showed dose-dependent reduction of the serum level of the tested biomarkers as compared to the IR group. This signifies the protective behaviour of RPC against IR injury.

### The effect of RPC on oxidative stress biomarkers

The effect of RPC was assessed on numerous biomarkers of oxidative stress, as presented in Figure 4. The level of MDA was found to be elevated, together with a reduction in SOD and GSH in the IR group, as compared with the sham group. Moreover, upon administration of RPC, the level of these biomarkers depicting oxidative stress was significantly restored approximately near to normal in a dose-dependent manner.

### The effect of RPC on the pro-inflammatory cytokines

As shown in Figure 5, the level of cytokines such as TNF-$\alpha$, IL-1$\beta$, and IL-6 was found to be markedly increased in the IR group as compared to the controls. However, the levels of these tested cytokines were found to be significantly reduced in the RPC-treated group in a dose-dependent manner. Thus, it could be suggested that RPC has

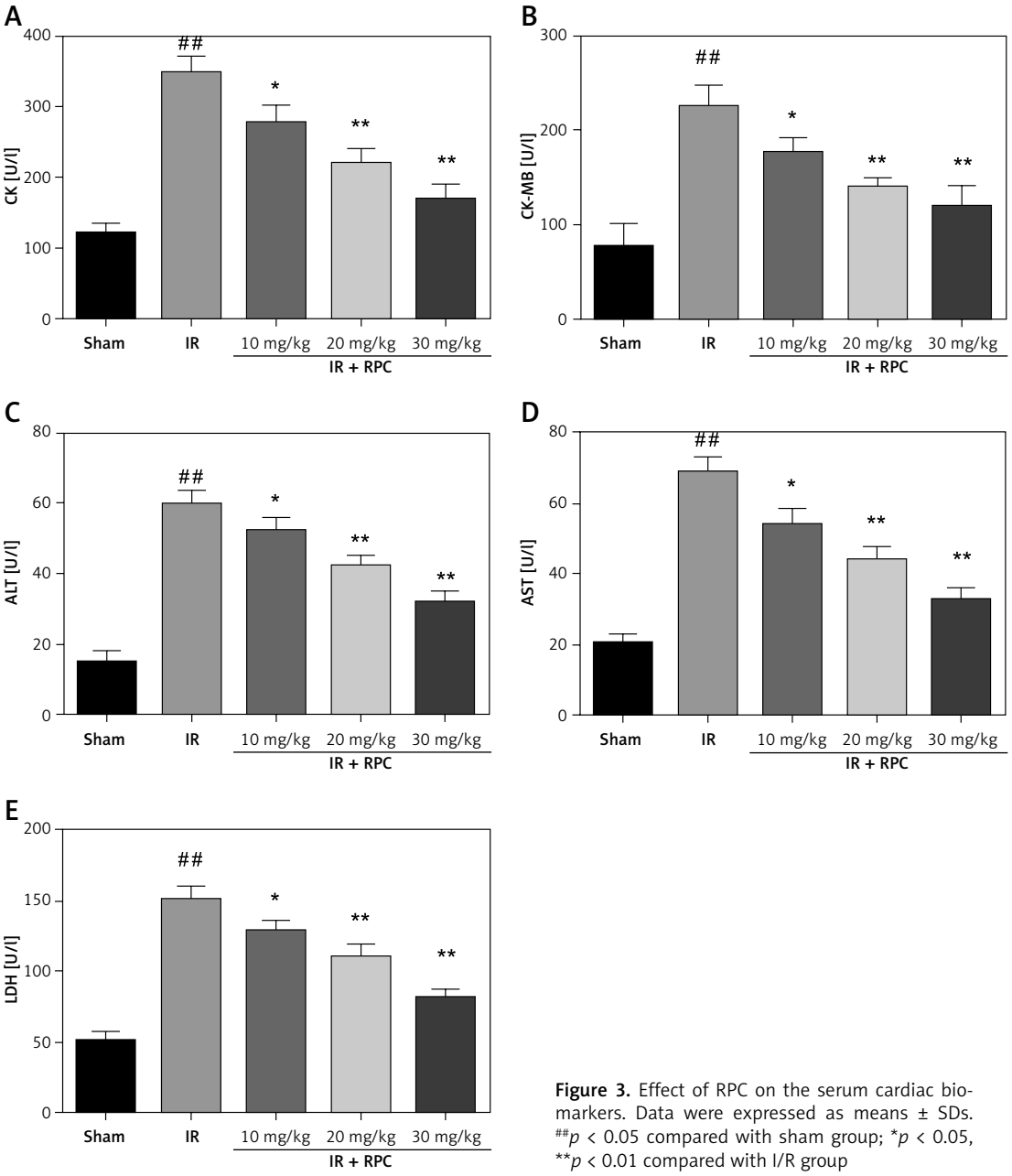


**Figure 3.** Effect of RPC on the serum cardiac biomarkers. Data were expressed as means ± SDs. $^{\#\#}p < 0.05$ compared with sham group; $^{*}p < 0.05$, $^{**}p < 0.01$ compared with I/R group

an inhibitory effect on the hyperactivated pro-inflammatory cytokines.

### The effect of RPC on cellular apoptosis

As shown in Figure 6, results suggested that, as compared to sham, the rate of cell apoptosis was found to be highly increased in the IR disease group, whereas in the RPC-treated group, the rate of cellular apoptosis was found to be greatly reduced.

### The effect of ropivacaine on COX-2 and iNOS by western blot analysis

As expected, the IR group with no treatment showed an elevated level of these 2 proteins in comparison to the sham. The RPC treated group, in a dose-dependent manner, showed a drastic drop in the level of COX-2 and iNOS (Figure 7). These results suggest that RPC might exert a cardioprotective effect via strong anti-inflammatory activity.

### Docking analysis of ropivacaine with COX-2

In docking analysis, RPC was found to be deeply buried into the active site of the COX-2 protein structure by interacting with key catalytic residues (Figure 8). To further elaborate these interactions, a 2D interaction diagram of RPC with COX-2 was generated and displayed in Figure 9. It was found that RPC creates numerous interactions with

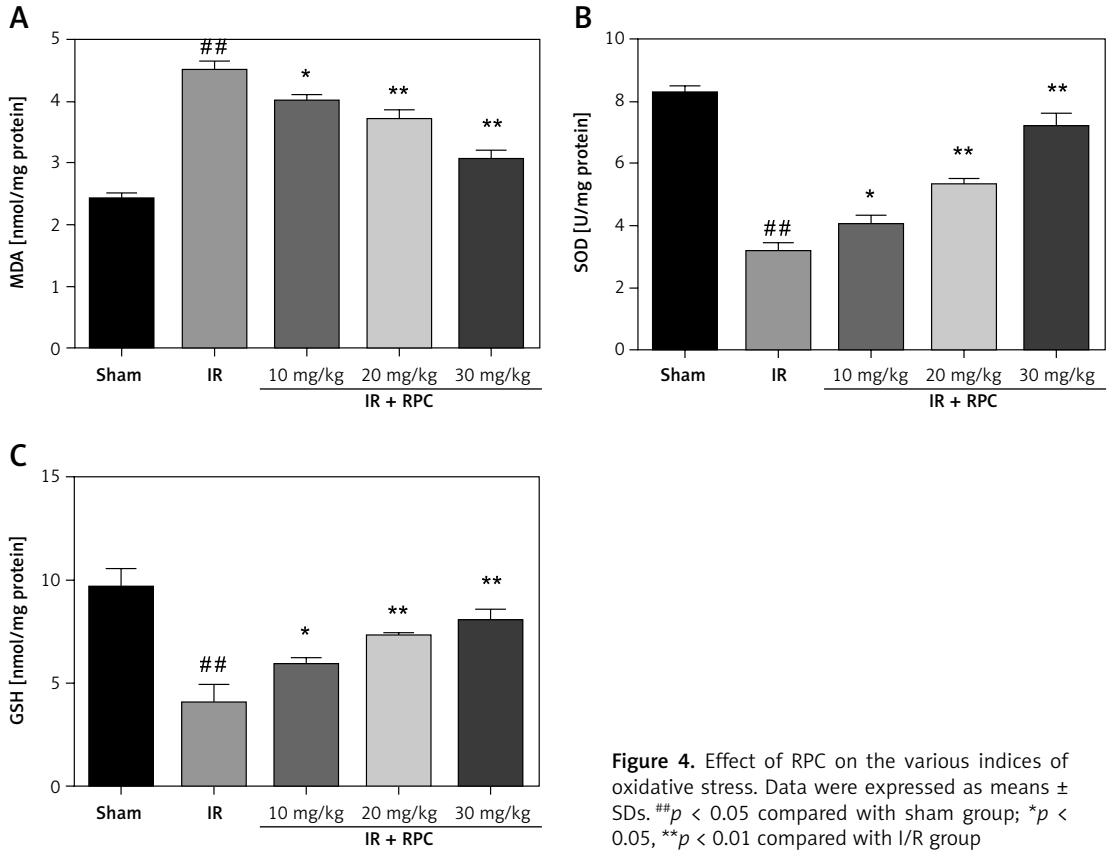


**Figure 4.** Effect of RPC on the various indices of oxidative stress. Data were expressed as means ± SDs. $^{\#\#}p < 0.05$ compared with sham group; $^{*}p < 0.05$, $^{**}p < 0.01$ compared with I/R group

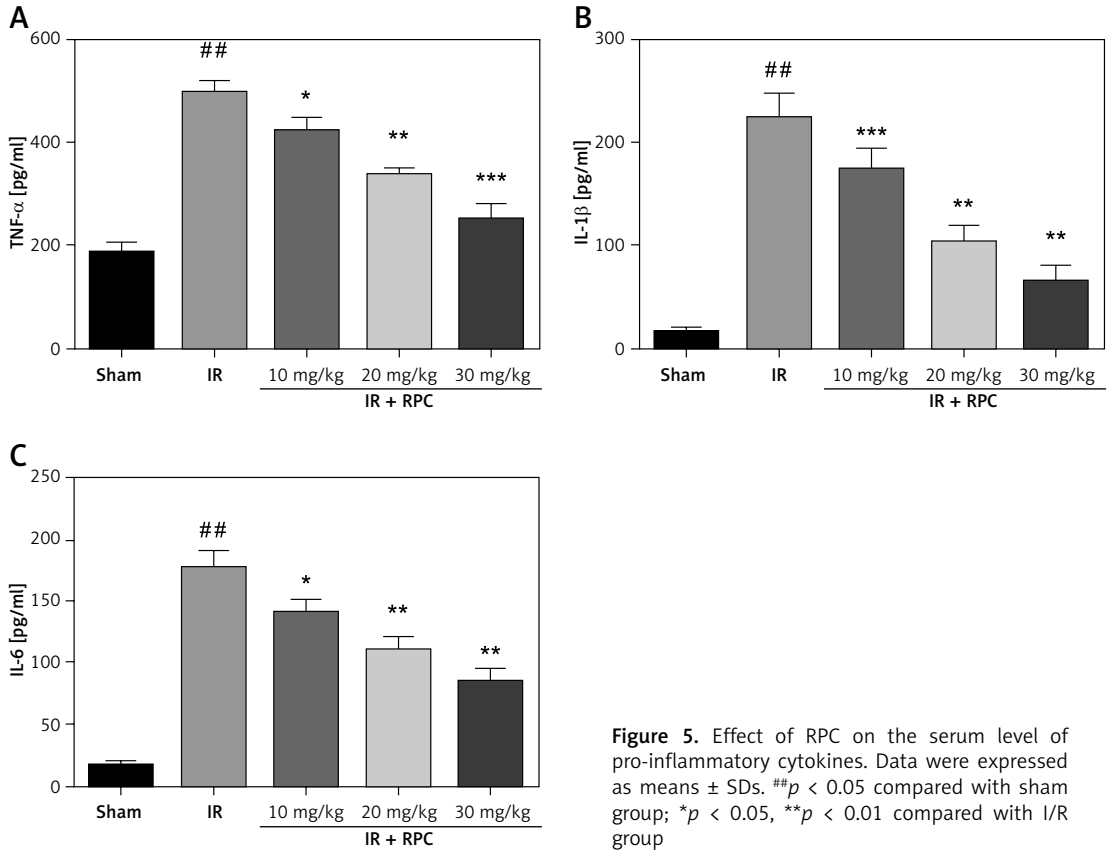


**Figure 5.** Effect of RPC on the serum level of pro-inflammatory cytokines. Data were expressed as means ± SDs. $^{\#\#}p < 0.05$ compared with sham group; $^{*}p < 0.05$, $^{**}p < 0.01$ compared with I/R group

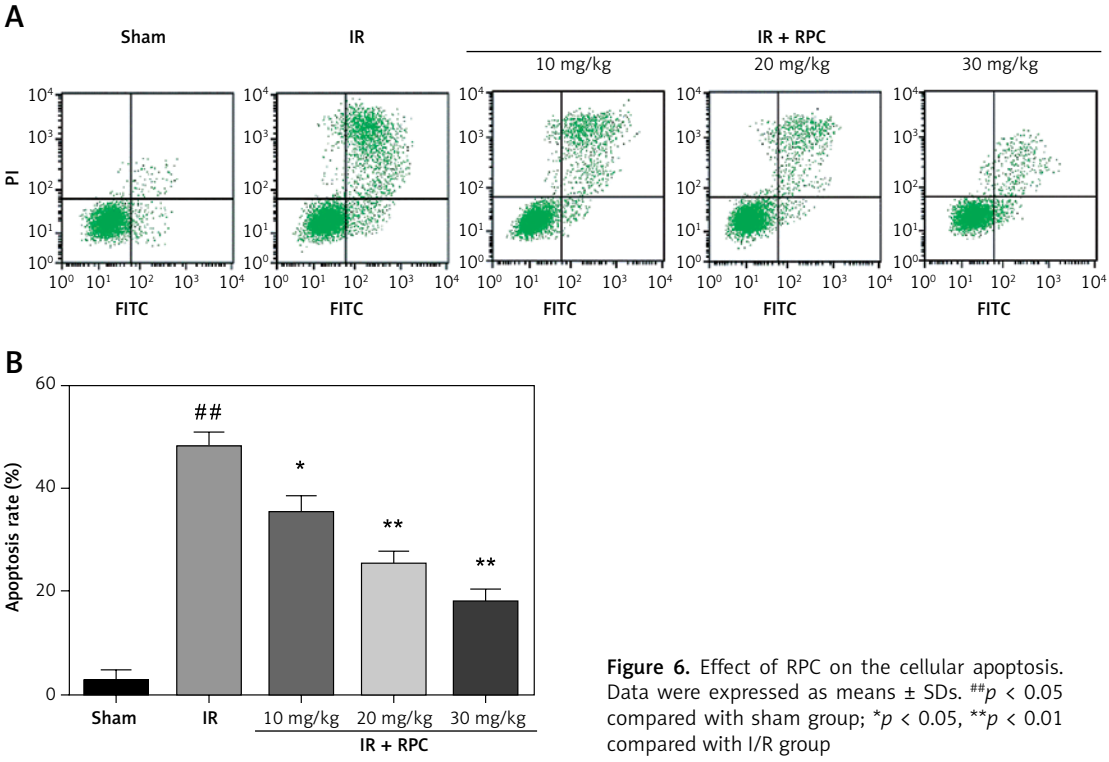


**Figure 6.** Effect of RPC on the cellular apoptosis. Data were expressed as means ± SDs. $^{\#\#}p < 0.05$ compared with sham group; $^{*}p < 0.05$, $^{**}p < 0.01$ compared with I/R group

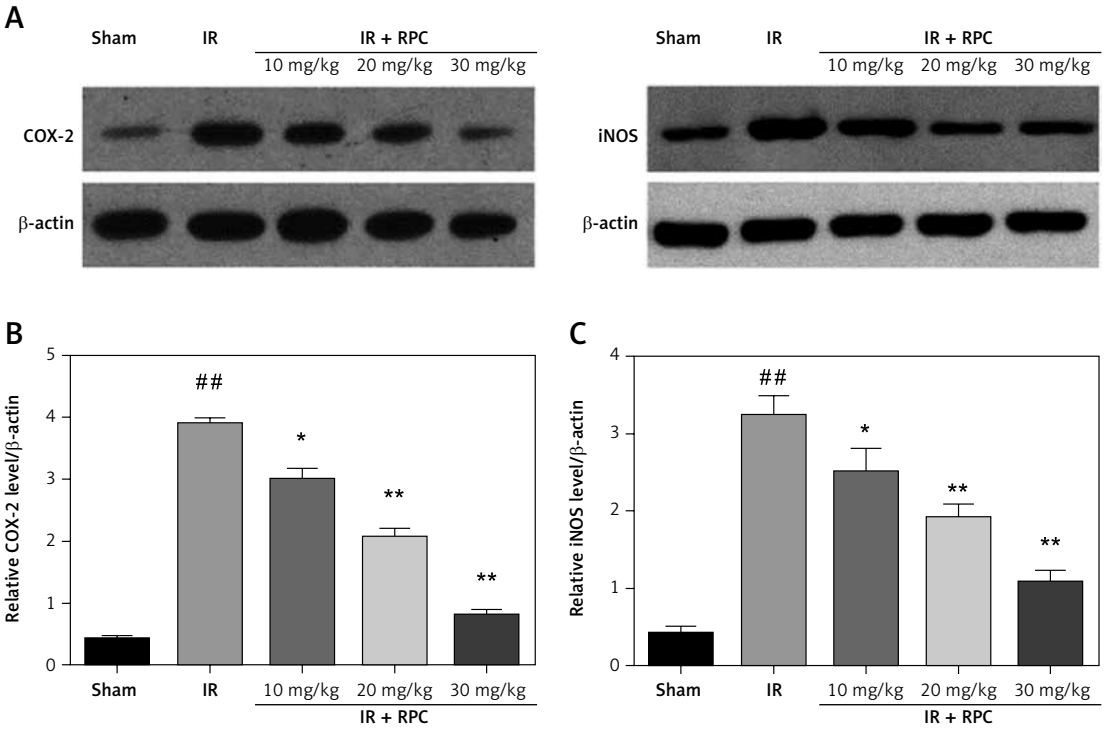


**Figure 7.** Effect of RPC on the expression of COX-2 and iNOS by western blot analysis. Data were expressed as means ± SDs. $^{\#\#}p < 0.05$ compared with sham group; $^{*}p < 0.05$, $^{**}p < 0.01$ compared with I/R group

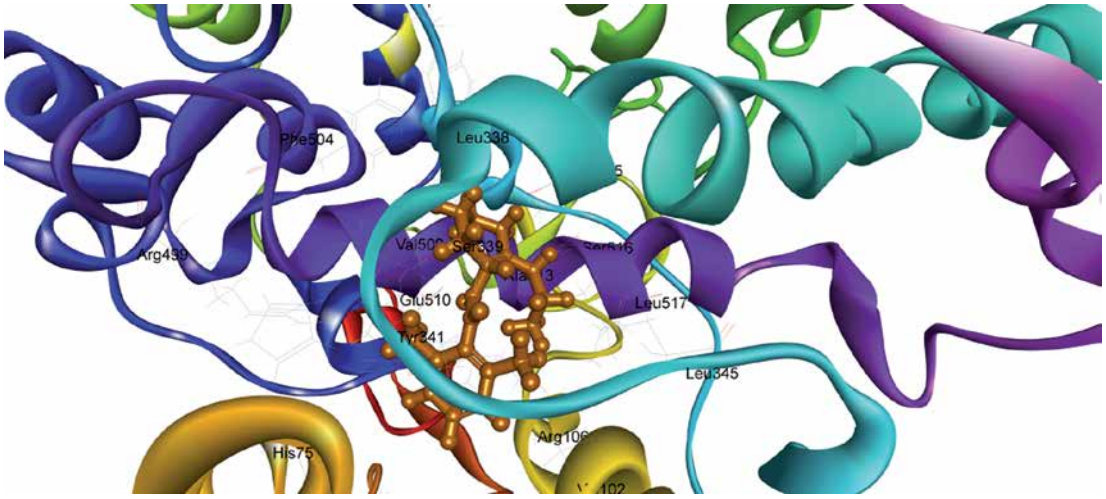


**Figure 8.** 3D orientation of RPC (shown in brown ball and stick) into the catalytic site of COX-2 (shown in ribbon)

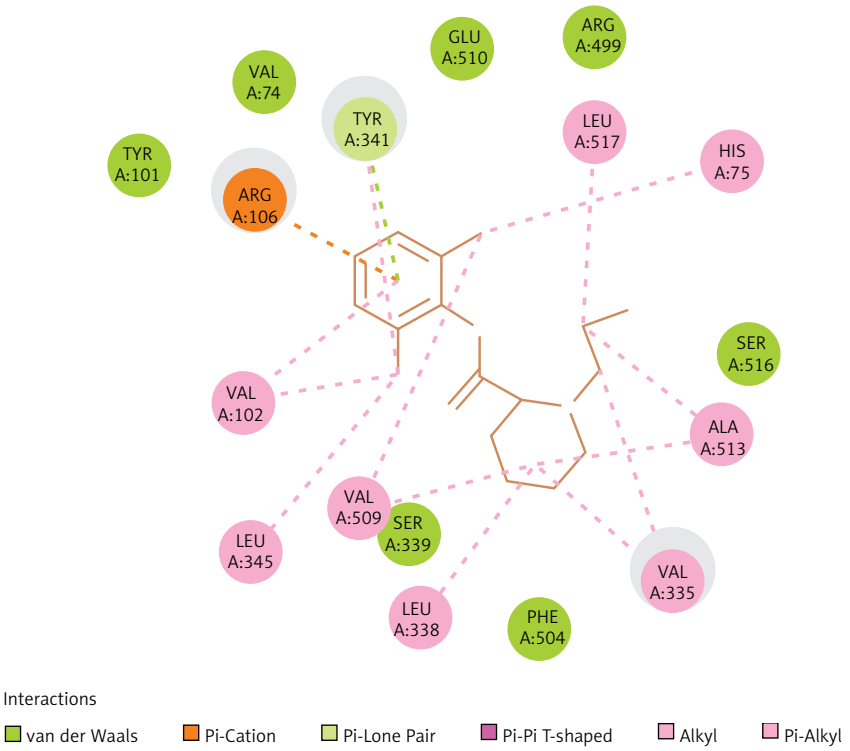


**Figure 9.** 2D orientation of RPC into the catalytic site of COX-2

neighbouring residues via the formation of many van Der Waals and pi-interactions with Tyr381, Arg106, Val102, Leu345, Val509, Ser339, Leu338, Val335, Ala513, His75, and Leu517. The interaction shown by RPC was found to be similar to previous studies [33]. These strong interactions of RPC with the catalytic site of COX-2 provide the basis for its inhibitory activity against COX-2.

## Discussion

Myocardial ischaemia/reperfusion injury is a devastating illness that impacts millions of individuals, either directly as a patient or indirectly as a caregiver or family member of an affected individual. It creates a huge economic burden on the patients and their families due to long hospital stays. Thus, new agents are continuously developed towards finding a cheap and economic way to deal with it. In our present study, we have successfully demonstrated the protective effect of ropivacaine against experimentally induced myocardial ischaemia/reperfusion injury in SD rats. We have selected male SD rats because extra oestrogen treatment may result in reduced

infarct size [34]. Secondly, accumulating evidence suggests that the infarct area in females is smaller than in males relative to body mass [35]. It has also been found that extra preconditioning and other cardio-protective approaches do not have any potential beneficial effect in females [36]. The interactions between cardioprotective signalling pathways in female animals and pre-and post-conditioning signalling pathways might be the reason for this effect. Infarction is the main prognostic factor in I/R injury. Studies showed that many novel agents provide a beneficial effect against IR injury via reduction of the infarct size. It has been shown that the infarct reducing the ability of any agent is directly correlated with its protective effect against myocardial IR injury [37–39]. Initially, the effect of RPC was investigated on the haemodynamic parameter of MI/R injury in rats. It was found that RPC restored the level of LVSP and ±dp/dt max, and LVEDP near to normal in a concentration-dependent manner. The ST-segment elevation is a characteristic hallmark of ischaemia/reperfusion injury, thus agents reducing ST-segment provide a beneficial effect against MI/R injury [40, 41]. In ECG analysis, RPC reduces ST-segment in rats. The effect of RPC was investigated on the infarct size of IR animals. It has been found that RPC significantly reduces infarct size. Accumulating evidence suggests that the utility of histopathological examination of myocardial tissue is a significant parameter for the assessment of the protection of chemicals against IR injury. It is a widely accepted methodology that allows the investigator to examine the cardiac tissue to assess the rate of improvement after the treatment [42–46]. Thus, we aimed to analyse the histopathology of cardiac tissues by H and E staining after administration of RPC to macroscopically visualize its cardioprotective effect. It was found that RPC causes improvement in the cardiac histopathology of animals. Cardiac injury biomarkers (LDH and MB) play a significant role in diagnostics, which allows the planning of a therapeutic regime during the time of injury or after the injury. It provides a suitable and efficient way to evaluate heart function. Compelling studies have suggested that after I/R injury the levels of these biomarkers were highly deregulated in direct proportion to the extent of I/R injury [47–49]. In the present study, RPC caused a significant reduction in cardiac injury biomarkers. Various evidence suggests that oxidative stress has a highly deleterious effect on the recovery from myocardial ischaemia/reperfusion injury. It induces the production of an excessive amount of reactive oxygen species to the impaired antioxidant system. These radicals are supposed to cause the annihilation of proteins, DNA, and lipids, simultaneously damaging the membrane to induce cell death [50–52]. Thus, agents improving the antioxidant system showed benefit against myocardial ischaemia/reperfusion. The RPC causes significant improvement of the antioxidant system after I/R injury, which was found following earlier studies. Inflammation in the hallmark of myocardial I/R injury. It is fuelled by the production of reactive oxygen species, which in turn promote oxidative stress and initiate a cascade of inflammatory response via recruitment of various pro-inflammatory cytokines [53, 54]. In the present study, RPC caused a reduction of the serum level of various pro-inflammatory cytokines. Myocardial apoptosis is greatly increased after IR injury and leads to the necrosis of myocardial tissue. Therefore, it is important to assess the effect of RPC on myocardial tissue [55, 56]. Towards this end, annexin V-FITC/PI double staining and flow cytometry was undertaken to analyse the effect of RPC on the apoptosis of myocytes, and it was found that RPC reduced apoptosis in a dose-dependent manner. To comprehend the underlying mechanism of the strong anti-inflammatory effect of RPC, which might be the reason for its protective effect against IR injury, we next aimed to investigate its effect on the expression of COX-2 and iNOS by western blot analysis. These were believed to play a vital role in the progression of inflammation after injury [57, 58]. It was found that RPC causes a drastic decrease in the level of COX-2 and iNOS. Docking is a widely recognized and powerful tool in drug discovery to define the probable structural contacts of ligands with the protein of interest. It accelerates the process of drug discovery and helps in the discovery and optimization and preclinical tests [59–63]. Therefore, to provide the basis for strong COX-2 inhibitory activity of RPC, it was docked onto the catalytic site of the 3D-crystal structure of COX-2. These strong interactions of RPC with the catalytic site of COX-2 provide the rationale for its inhibitory activity against COX-2. Further research is required to investigate these in larger, longer-term studies.

In conclusion, our study has demonstrated the usefulness of ropivacaine against myocardial ischaemia/reperfusion injury. Ropivacaine showed a protective effect against myocardial injury possibly via amelioration of oxidative stress and apoptosis of myocardial tissue. Ropivacaine reduces inflammation in animals possibly via strong inhibition of COX-2 and iNOS. However, more studies are needed to provide the clinical basis of ropivacaine in myocardial injury.

## Conflict of interest

The authors declare no conflict of interest.

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