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研究室で撮影した本人のスナップ写真、及び発表論文のコピーを添付

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研究テーマ Nicorandilの心筋保護に関する研究

2．本年度の研究業績

(1) 学会・研究会等においての口頭発表 有 ・ (無) (学会名・内容)

(2) 学会誌等に発表した論文 (有) ・ 無 (雑誌名・論文名)

雑誌名: The Japanese Journal of Thoracic and Cardiovascular Surgery

論文名: ① Nicorandil pretreatment and improved myocardial protection during cold blood cardioplegia

② Cardioprotection Effect of Orally Administered Angiotensin-Converting Enzyme Inhibitor Against Ischemia-Reperfusion Injury in the Isolated Rat Heart

3．今後の研究計画

心筋保護の研究は心臓手術、心臓移植、心筋梗塞の内科介入治療に対して大変重要なことであり、これから心筋保護に関する研究を続きたいと思います。また最近注目されている経食道心エコー（TEE）も臨床で研究したいと思います。

4．研究指導者の意見

李さんは東北大学医学部の大学院生として心筋保護に関する研究に対して熱心を持って一生懸命にがんばってニコランジルの心筋保護効果に関する臨床研究は"日本胸部心臓血管外科雑誌に発表しました。また実験研究は The Journal of Surgical Research に投稿しています。そのほかに経食道心エコーをして手術中に投与しました。今後李さんは中国で研究や臨床活動の輝きを楽しみしています。

研究指導者氏名　　印

5．研究報告

別紙形式を参考に、報告本文4000字以上で報告して下さい（枚数自由・ワープロ使用）

タイトル・要旨等は日本語で、KEY WORDS以下は日本語或いは英語で記入して下さい。

研究成果の発表予定がある場合は発表原稿・抄録集等を添付して下さい。

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ワーキングモードを用いたラット摘出心の虚血のおける K^+カルディオプレジアに対するニコランジル前処置の優れた心筋保護作用

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要旨

本研究の目的は，１）ニコランジル前処置（ニコランジルの虚血直前投与）の心筋保護効果に関し，投与量と投与時期を検討すること，２）ニコランジル前処置の心筋保護効果をクリスタロイド心停止液と比較することである。

５４匹のラット（Wistar rat，250~350g）を使用し，摘出心をランゲンドルフ潅流装置に装着した。３７℃で２８分間潅流後（ランゲンドルフモード１０分間，ワーキングモード１０分間とランゲンドルフモード 8 分間），２８℃で６０分間の虚血とした。再潅流は３７℃で４５分間施行した（ランゲンドルフモード１５分間，ワーキングモード３０分間）。　５４匹のラットを無作為に以下の９グループ（各グループ，n = 6）に分類した：コントロール群，ニコランジル前処置群［投与量により４群に分類した：10 μmol/l 投与群— Pre-NC(10)，30 μmol/l 投与群— Pre-NC(30)，100 μmol/l 投与群— Pre-NC(100)，300 μmol/l 投与群— Pre-NC(300)］，クリスタロイド心停止液群（CP），ニコランジルプレコンディショニング群（ニコランジルの虚血５分前投与群— NC-P），Pre-NC(30) + CP 群，　NC-P + CP 群。　虚血前後に心拍出量(CO)，冠血流量(CF)をもとめ各群間で比較検討した。また，虚血後心停止までの時間をもとめ各群間で比較検討した。全ての値は，平均値±標準誤差で表し，比較は分散分析，Post hoc テストを用いて施行した。　P 値が 0.05 以下で有意差ありとした。

Pre-NC(30) および Pre-NC(100) 群の CO と CF のパーセント回復率はコントロール群に比して有意に良好であった(%CO:62.9±1.8, 48.9±3.1 vs 34.3±2.9, P<0.05, %CF : 73.0±3.5, 59.7±2.9 vs 44.3±3.6, P<0.05)。Pre-NC(300) 群の CO と CF のパーセント回復率はコントロール群に比して不良であった (%CO: 16.1±6.0 vs 34.3±2.9, %CF: 28.5±9.6 vs 44.3±3.6, P<0.05)。Pre-NC(30) および Pre-NC(100) 群の虚血後心停止までの時間はコントロール群に比して有意に短かかった (8.8±0.2 分, 11.1±2.9 分 vs 20.3±1.6 分, P<0.05)。Pre-NC(10) および Pre-NC(300) 群の虚血後心停止までの時間はコントロール群と差がなかった。Pre-NC(30) 群の CO と CF のパーセント回復率は CP 群および NC-P 群に比して有意に良好であった (%CO : 62.9±1.8 vs 46.5±4.6, 46.1±1.6, P<0.05, %CF : 73.0±3.5 vs 55.0±7.2, 59.0±2.9, P<0.05)。 Pre-NC(30) + CP 群の CO パーセント回復率は CP 群および NC-P 群に比して有意に良好であった(%CO:68.4±1.7 vs 46.5±4.6, 46.1±1.6, P<0.05)。 しかし, Pre-NC(30) + CP 群の CF のパーセント回復率は CP 群および NC-P 群と差がみられなかった (%CF : 63.9±3.3 vs 55.0±7.2, 59.0±2.9)。NC-P + CP 群の CO と CF のパーセント回復率は NC-P 群と差がなかった。

結論 : 1) 30 μmol/l 群のニコランジル前処置が最も良好な心筋保護効果を呈した。2) ニコランジル前処置 [Pre-NC(30) 群] はクリスタロイド心停止液より良好な心筋保護効果を呈した。3) ニコランジル前処置 [Pre-NC(30) 群] はニコランジルプレコンディショニング群より良好な心筋保護効果を呈した。

以上より低濃度のニコランジルの虚血直前投与は良好な心筋保護効果を有することが判明し, 今後の臨床の可能性も持ちと考える。

Superior Cardioprotective Effect of Nicorandil Pretreatment to Potassium Cardioplegia during Global Ischemia in Isolated Working Rat Hearts

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Abstract

Objective: This study was designed to 1) elucidate the dose-response relationship between nicorandil pretreatment (nicorandil administration just before ischemia) and cardioprotection, 2) compare the effect of nicorandil pretreatment with nicorandil preconditioning (nicorandil administration followed by 5 minutes of drug-free perfusion before ischemia) and potassium cardioplegia against ischemia/reperfusion injury. **Methods:** Fifty-four isolated working Wistar rat hearts underwent 28 minutes of 37℃ equilibrium (Langendorff-mode and working-mode), 60 minutes of 28℃ global ischemia, followed by 45 minutes of 37℃ reperfusion (Langendorff-mode and working-mode). Cardiac function was assessed at the end of working-mode. **Results:** Nicorandil pretreatment (30 and 100 μmol/l) significantly improved the post-ischemic percent recovery of cardiac output and coronary flow (%CO: 62.9±1.8 and 48.9±3.1 versus 34.3 ±2.9 in the control, %CF: 73.0±3.5, 59.0±2.9 versus 44.3±3.6 in the control, $P < 0.05$ respectively). Furthermore, nicorandil pretreatment showed superior cardioprotection to potassium cardioplegia (%CO: 46.5±4.6, %CF: 55.0±7.2) and nicorandil preconditioning (%CO: 46.1±1.6, %CF: 59.0±2.9). Nicorandil pretreatment with potassium cardioplegia showed superior %CO (68.4±1.7) to potassium cardioplegia and nicorandil preconditioning, however it did not show better %CF (63.9±3.3) than potassium cardioplegia or nicorandil preconditioning. **Conclusions:** These data demonstrate (1) nicorandil pretreatment (30 μmol/l) shows the maximal cardioprotective effect, (2) nicorandil pretreatment provides superior cardioprotection to potassium cardioplegia, (3) nicorandil pretreatment is more efficient than nicorandil preconditioning.

Key words: cardioprotection; nicorandil; potassium cardioplegia; ischemia/reperfusion injury.

1. Introduction

Cardioprotection is a major concern in ischemic/reperfusion injury such as thrombolytic therapy for acute myocardial infarction, open heart surgery and

cardiac transplantation. Potassium cardioplegia (CP) has been widely used as a conventional method of cardioprotection [1]. Potassium at high concentration depolarizes the myocardial cell membrane associated with rapid electromechanical arrest, and markedly reduces cellular energy expenditure during the ischemic period. However, depolarization of the membrane is also associated with that the exchange of intracellular sodium for calcium via the sodium-calcium exchange, influx of calcium through the calcium " window current" and derangement in transmembrane ion pumps [2]. These factors contribute to calcium overload and depletion of energy store, which is related to irreversible myocardial ischemic damage and reperfusion injury [3,4].

The natural resting state of the myocardial cell is at hyperpolarized membrane potentials [5], in which transmembrane ion gradients are balanced and energy requirements are minimal. ATP-sensitive potassium channels opener hyperpolarizes the membrane of myocyte and has been defined as "hyperpolarizing cardioplegia" [2,6] – a novel form of myocardial protection.

Nicorandil (2-nicotinamidoethyl-nitrate, NC) has drawn attention as a hybrid between an ATP-sensitive potassium channel opener and a nitrate [7]. It is suggested that the myocardial protection of NC is induced by its direct effect through the activation of ATP-sensitive potassium channels [8,9]. Opening ATP-sensitive potassium channel are responsible for an outward potassium current that shortens action potential and decreases the potential-operated calcium channel opening [10]. The reduction of calcium overload and decline in contractile activity restrict the consumption of intracellular ATP in the ischemic myocyte, which is beneficial against ischemia/reperfusion injury [11,12]. Recently subsequent evidence also suggested that the ATP-sensitive potassium channel in the

mitochondrial membrane (mito K_{ATP}) is the mediator of cardioprotection [13-15]. The effect may occur as the result of K^+ entry and intramitochondrial depolarization, which would reduce mitochondrial calcium overload leading to enhance ATP synthesis [16,17]. On the other hand, when ATP-sensitive potassium channel is opened, the membrane of coronary vessel endothelial cell is hyperpolarized and the concentration of the intracellular calcium is decreased, which contributes to vasodilation of the smaller coronary vessel [18]. The nitrate-like property of NC actives the vessel smooth muscle and reduces NO, which leads to the vasodilation of the larger coronary vessel [19]. Thus the increase of coronary flow provides an additional beneficial effect on the recovery of post-ischemic cardiac function [20].

NC has been used clinically for patients with ischemic heart disease and had been demonstrated to improve cardioprotective effect as an additive to potassium cardioplegia [21,22] or be efficient as same as CP [23]. However, there have been few investigations of NC as a novel cardioplegic agent to alternate the traditional cardioplegic method and whether the cardioprotection of NC pretreatment is better than CP. In this study, with isolated working rat hearts during 60 minutes 28℃ global ischemia, we designed to compare the effect of NC pretreatment as a cardioplegia agent against ischemia/reperfusion injury with CP, to elucidate dose-related toxicity, to examine the administration time and test the effect of NC combined with CP.

2. Materials and methods

2.1. Preparation of isolated working hearts.

This study was approved by Committee of Animal Experimentation of Tohoku University School of Medicine (in reference to "The Classification of Biomedical Experiments Based on Ethical Concerns for Non-human species", Laboratory Animal Science, Special Issue P.14-16, 1987). The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Fifty-four adult male Wistar rats (250-350 g) were anesthetized with sodium pentobarbital (60 mg/kg) and heparinized (300 IU) with an intraperitoneal injection. The heart was excised and immediately immersed in ice-cold Krebs-Henseleit bicarbonate (KHB) buffer, consisting of: NaCl (118.5 mmol/l), KCl (4.7 mmol/l), $CaCl_2$ (2.5 mmol/l), $MgSO_4$ (1.2 mmol/l), KH_2PO_4 (1.2 mmol/l), $NaHCO_3$ (25.0 mmol/l) and glucose (11.0 mmol/l). Then, the heart was mounted on a Langendorff apparatus (IPH-W, S/N 300497, Labo Support Corporation, Osaka, Japan) via the aorta, and perfused with KHB solution at a constant pressure of 70 mmHg. KHB solution was equilibrated with 95% oxygen and 5% carbon dioxide ($PO_2 > 500$ mmHg, PH = 7.42±0.05), and maintained at 37℃ with a water jacket. During 10-minutes of non-working Langendorff mode (L-mode), the left atrium was cannulated. Then the heart was switched to working mode (W-mode) with a left atrium pressure of 13 mmHg and an aortic pressure of 70 mmHg [24]. All hearts were paced at a constant rate of 300 beats/min during the baseline and reperfusion period.

2.2. Experimental groups.

Fifty-four rat hearts were randomly distributed to nine groups (n=6 in each

group, Table.1). Control hearts received a perfusion with 8 minutes of KHB buffer before global ischemia without NC or CP. In the NC pretreatment group (Pre-NC), NC was administrated for 3 minutes just before ischemia; different dose (10, 30, 100 and 300 μmol/L) of NC was pretreated in 4 Pre-NC groups: Pre-NC(10), Pre-NC(30) Pre-NC(100) and Pre-NC(1/300). In the NC preconditioning group (NC-P), NC was administrated for 3 minutes followed by 5 minutes of drug-free KHB perfusion before ischemia. In the CP group, CP was given during the first minute of global ischemia. In the Pre-NC+CP group, NC pretreatment combined with CP administrated. And in the NC-P+CP group, NC preconditioning combined with CP was administrated.

NC was dissolved in KHB immediately and filtered through a 0.45 μm porosity membrane before use, then was delivered into the aortic retrograde perfusion circuit for 3 minutes to achieve a different dose (10, 30, 100 and 300 μmol/L). The range of concentration chosen to test the cardioprotection of NC was based on previous studies [22, 25]. NC was provided by Chugai Pharmaceuticals (Tokyo, Japan).

The CP solution was prepared by potassium chloride (KCl) added to the KHB buffer (potassium concentration: 20 mmol/l) and infused through a side arm of the aortic cannula at a pressure of 45 mmHg and at 28℃ during the first minute of global ischemia. The CP solution was filtered before use through a 0.45 μm porosity membrane and gassed with 95% oxygen and 5% carbon dioxide. During the ischemic period, the myocardial temperature was kept at 28℃ in a water jacket bath.

2.3. Experimental protocol.

All the hearts underwent 28 minutes of 37℃ equilibrium (10 minutes of L-mode, 10 minutes of W-mode and 8 minutes of L-mode, NC administration when applied), then 60 minutes of 28℃ global ischemia (CP administration when applied), followed by 45 minutes of 37℃ reperfusion (15 minutes of L-mode and 30 minutes of W-mode) (Fig.1).

The post-ischemic recovery of cardiac function and the time to contractile arrest after ischemia of Pre-NC at different concentrations (10, 30, 100 or 300 μ mol/l) was designed to elucidate. And the post-ischemic recovery of cardiac function among the CP and Pre-NC, NC-P administrated alone or combined with CP was designed to compare. On the base of the dose-response study, 30 μmol/l dose of NC was selected

2.4. Function measurement.

Aortic flow (AF), coronary flow (CF) were measured at the end of W-mode before global ischemia and at the end of reperfusion. The AF (ml/min) was measured by an electromagnetic flow meter (Nihon Kohden, Tokyo, Japan) positioned in the aortic outflow line, and CF (ml/min) was measured by timed collection of the coronary venous effluent. Cardiac output (CO, ml/min) was calculated as the sum of AF and AF. Post-ischemic recovery of cardiac function was expressed as percent to pre-ischemic values in each heart (%CO, %CF). The time to contractile arrest (minute) during ischemia was measured after global ischemia.

2.5. Statistical analysis.

All cumulative results were expressed as a mean ± standard error of the mean. Analysis of variance and Post hoc test followed by Fisher's Protected Least Significant Difference were used to compare mutually exclusive data among the groups when appropriate. A difference was considered statistically significant when $P < 0.05$.

3. Results

3.1. Recovery of cardiac function in the dose-response study of Pre- NC

The post-ischemic percent recovery of cardiac function (%CO and %CF) in the dose-response study of Pre-NC was demonstrated in Fig.2. Significantly better %CO and %CF were observed in the hearts that received Pre-NC at concentration of 30 and 100 μmol/l than control hearts (%CO: 62.9$\pm$1.8 and 48.9 $\pm$3.1 versus 34.3$\pm$2.9 in the control; $p<0.05$, %CF: 73.0$\pm$3.5, 59.7$\pm$2.9 versus 44.3$\pm$3.6 in the control; $p<0.05$). The %CO and %CF in the hearts receiving a 300 μmol/l dose of NC (%CO: 16.1$\pm$6.0, %CF: 28.5$\pm$9.6) were lower than that in the control hearts ($p<0.05$). There was no statistically significant difference of the recovery of cardiac function in the hearts receiving 10 μmol/l dose of NC with the control hearts.

3.2. Time to cardiac arrest in the dose-response study of Pre- NC

The time to cardiac mechanical arrest after ischemia in the dose-response study of Pre-NC was shown in Fig.3. Pre-NC at doses of 30 and 100 μmol/l significantly accelerated the time (minute) to cardiac mechanical arrest (8.8$\pm$ 0.2and 11.1$\pm$2.9 versus 20.3$\pm$1.6 in the control; $p<0.05$). Pre-NC at doses of 10 and 300 μmol/l had no significant difference when compared with the control group.

3.3. Comparison of the recovery of cardiac output in the six groups

The post-ischemic percent recovery of cardiac output (%CO) among the control, CP and Pre-NC, NC-P administrated alone or combined with CP groups was summarized in Fig.4. A significantly better %CO was observed in the hearts that received CP and Pre-NC, NC-P administrated alone or combined with CP (46.5 ±4.6 and 62.9±1.8, 68.4±1.7, 46.1±1.6, 44.6±1.6 versus 34.3±2.9 in the control; $p<0.05$). Furthermore, Pre-NC administrated alone or combined with CP showed superior %CO to CP and NC-P administrated alone or combined with CP ($p < 0.05$). And there was no statistically significant difference of the %CO among the hearts that underwent CP and NC-P administrated alone or combined with CP.

3.3. Comparison of the recovery of coronary flow in the six groups

The post-ischemic percent recovery of coronary flow among the control, CP and Pre-NC, NC-P administrated alone or combined with CP groups was summarized in Fig.5. A significantly better %CF was observed in the hearts that received Pre-NC administrated alone or combined with CP and NC-P groups (73.0 ±3.5, 63.9±3.3 and 59.0±2.9 versus 44.3±3.6 in the control; $p<0.05$). There was no statistically significant difference of the %CF among the hearts that underwent CP (55.0±7.2), NC-P administrated combined with CP (53.2±2.9) and the control hearts. Furthermore, Pre-NC also showed superior %CF to CP and NC-P administrated alone or combined with CP ($p < 0.05$). However, there was no statistically significant difference of the %CF among the hearts that underwent Pre-NC administrated combined with CP, CP and NC-P administrated alone or combined with CP.

4. Discussion

This study has demonstrated that NC pretreatment as a cardioplegic agent against ischemia/reperfusion injury provided a superior post-ischemic recovery of cardiac function to CP. Furthermore, NC pretreatment significantly increased the post-ischemic percent recovery of coronary flow, which results support the mechanism link with vasodilatation of coronary arteries by its ATP-sensitive and nitrate-like properties, leading to an additional beneficial effect on cardioprotection. This data is consistent with a previous obstruction that EDHF (endothelium-derived hyperpolarization factor) nmediated coronary endothelial function is maximally preserved by hyperpolarizing cardioplegia but impaired by depolarizing cardioplegia [26] .

There has been no previous investigation of NC as a cardioplegic agent to show superior cardioprotective effect to traditional CP. Only one recent study had shown improvement of functional recovery of NC (100 μmol/l and 1 μmol/l) as effective as CP using a blood perfusion isolated rabbit heart during normothermic global ischemia [23] . However, blood cardioplegic solutions have been repeatedly shown to be more protective of post-ischemic function than crystalloid cardioplegia solutions. One can thus hypothesize that blood cardioplegia might be more protective of the hyperpolarized myocardium than the depolarized myocardium. In this way, administering the hyperpolarized cardioplegia-NC in a sanguineous cardioplegic solution might result in improved recovery of cardiac function against ischemia/reperfusion injury when compared with the depolarized cardioplegia-Potassium cardioplegia. Further investigation is necessary to define the possibility.

A puzzling observation in this study is that NC pretreatment significantly improved the recovery of cardiac function in comparison with NC preconditioning. Regardless of the exact mechanism by which NC preconditioning mimics ischemic preconditioning to produce the cardioprotective effect [27-29], the mechanism by which the heart keeps the "memory" of its brief exposure to NC is yet unknown, but has been speculated that NC sustains opening of partial potassium channels and decreases the threshold for channel activation during the ensuing period of protracted ischemia [9], which would result in a limitation with the cardioprotection of NC preconditioning.

In the present data, NC pretreatment combined with CP significantly improved the recovery of post-ischemic cardiac output which was almost the same as NC pretreatment alone, however, it did not show a better recovery of post-ischemic coronary flow than CP. The mechanistic link might be that vasodilation of coronary artery of hyperpolarizing cardioplegia-NC is abolished followed by administration of CP which depolarizes the membrane of coronary vessel endothelial cell leading to the increase of concentration of the intracellular calcium and causing systolic of coronary vessel [26]. Therefore it contributes to reduce recovery of post-ischemic coronary flow.

One previous study using the isolated rat hearts reported that NC pretreatment at doses of 300 μmol/l improved the recovery of the contractile function after 25 minutes of normothermic global ischemia [19]. Another experiment showed 30 μmol/l to 3 mmol/l of NC increased the recovery of cardiac function after 80 minutes of 20℃ global ischemia [30]. However, in this study, we used 28℃ global ischemia which is a relatively lower temperature and considered to be a commonly used cardioprotective method during open-heart operation. We found

that NC pretreatment 100 μmol/l had a significant improvement of post-ischemic recovery of cardiac function, and a more detail dose-dependent study showed a lower dose of NC 30 μmol/l provided a maximal cardioprotective effect. In addition, a negative inotropic effect with ventricular arrhythmias happened when using NC equal to or higher than 300 μmol/, which is consistent with several studies that showed high doses of NC exert a negative inotropic effect with arrhythmias [31,32]. The reason may be related to the chemical structure of NC and a higher dose of NC being a factor to influence the activation of ATP-sensitive potassium channel.

At the same time, NC pretreatment induced a significantly shortened time to mechanical arrest that tended to be similar to that of CP. That the time to mechanical arrest by NC pretreatment was shortened seems to be a direct result of ATP-sensitive potassium channel opening; the addition of glibenclamide to NC completely abolished the ability of NC [23].

Our previous clinical study showed that 0.3 mg/kg (100 μmol/l) NC which administrated 30 minutes before aortic clamp combined with CP tended to increase the recovery of cardiac output and significantly decreased release of cardiac enzymes after cardiac arrest when compared with CP during the open-heart operation [33]. This animal experiment provides an additional reference about the dose and timing of the addition of NC to clinical study. Whether NC pretreatment with 30 μmol/l just before aortic clamp providing a superior cardioprotection to CP is among the possibility that warrant further investigation.

In summery, the present study has demonstrated that NC is a superior cardioprotective agent against ischemia/reperfusion injury to CP with isolated working rat hearts during 60 minutes 28℃ global ischemia. Among the

relatively wide therapeutic range of NC, there is a maximal concentration (30 μ mol/l), and NC pretreatment just before ischemia as its administration time provides more effective cardioprotection. This result suggests that nicorandil pretreatment may be an alternative to traditional cardioprotection in open-heart surgery.

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Figure Legend

Fig.1. Experimental protocol. L-M, Langendorff-mode; W-M, Working-mode; Pre-NC (NC pretreatment just before ischemia); CP, potassium cardioplegia; NC-P (NC-preconditioning, NC administration followed by 5 minutes drug-free reperfusion); Pre-NC+CP, NC pretreatment in combination with CP; NC-P+CP, NC preconditioning in combination with CP.

Fig.2. Post-ischemic percent recovery of cardiac function (%CO and %CF) in the dose-response study of NC pretreatment (10, 30, 100 and 300 μmol/l). Values represent the mean±SEM. * $P < 0.05$ vs. control group.

Fig.3. Time to cardiac mechanical arrest (minute) after global ischemia in the dose-response study of NC pretreatment. Values represent the mean±SEM. * $P < 0.05$ vs. control group.

Fig.4. Post-ischemic percent recovery of cardiac output (%CO) among control, CP, Pre-NC, Pre-NC+CP, NC-P and NC-P+CP groups. Values represent the mean±SEM. * $P < 0.05$ vs. control group; $^{+}$ $P < 0.05$ vs. CP group; $^{\neq}$ $P < 0.05$ vs. NC-P group; $^{\#}$ $P < 0.05$vs. NC-P+CP group.

Fig.5. Post-ischemic percent recovery of coronary flow (%CF) among control, CP, Pre-NC, Pre-NC+CP, NC-P and NC-P+CP groups. Values represent the mean± SEM. * $P < 0.05$ vs. control group; $^{+}$ $P < 0.05$ vs. CP group; $^{\neq}$ $P < 0.05$ vs. NC-P group; $^{\#}$ $P < 0.05$vs. NC-P+CP group.