Effects of n-acetylcysteine on ischemic preconditioning. Study in isolated rat hearts

Efeitos da N-acetilcisteína no precondicionamento isquêmico: estudo em corações isolados de ratos

Denoel Marcelino de OLIVEIRA[1], Eros Silva GOMES[2], Tofy MUSSIVAND[3], Alfredo Inácio FIORELLI[4], Otoni Moreira GOMES[5]

Abstract

Objective: The aim of this study is to assess if N-Acetylcysteine (NAC) changes the Ischemic Preconditioning (IP) in isolated rat hearts using only one cycle of IP.

Methods: Heart Rate (HR), Coronary Flow (CF) and Myocardial Contractility (dP/dt) were registered in 30 Wistar rat's hearts. After anesthesia the hearts were removed and perfused with Krebes-Hensleit equilibrated solution with 95% of O_2 and 5% of CO_2 according Langendorff's method. GI: Control (n=6); GII: 20 min. ischemia (n=6); GIII: IP (n=6); GIV 50 µg/ml/min NAC before IP (n =6); GV: 100 µg/ml/min NAC before IP (n=6). Parameters were measured after 15 min. of stabilization (T 0) and T3, T5, T10, T15, T20, T25 and T30 min. after reperfusion. Statistical significance was considered when *P*<0.05.

Results: There were changes on HR comparing GI with GII at T20 and T25 and comparing GI with GIII, GIV with GV at T10 and T20 (*P*<0.05). CF was different comparing GI with GII at T3 and T5, GI with GIV at T10 and GI with GV at T10 and T25 (*P*<0.05). Myocardial Contractility was similar comparing GIII with GI and GV. GIII had higher dP/dt than

GIV but without statistical difference (*P*>0.05). dP/dt was higher in GV than GIV but with statistically significant difference only at T30.

Conclusion: dP/dt was better in preconditioned hearts and was changed if using NAC in GIV. The use of NAC didn't change the effects of preconditioning on myocardial contractility in GV.

Descriptors: Ischemic preconditioning. Myocardial infaction. Myocardial contraction. Free radicals. Animal experimentation.

Resumo

Objetivo: Avaliar se a N-Acetilcisteína (NAC) altera o Precondicionamento Isquêmico (PC) em corações isolados de ratos usando apenas um ciclo de PC.

Métodos: Freqüência Cardíaca (FC), Fluxo Coronariano (FLC) e Contratilidade Miocárdica (dP/dt) foram registradas em 30 corações de ratos Wistar. Após anestesia, os corações foram perfundidos em sistema de Langendorff com solução de Krebs-Hensleit (K-H), equilibrada (95% de O_2 e 5% de

1. Master's Degree in Cardiovascular Surgery; Cardiovascular Surgeon.
2. Specialist in Cardiology by BSC; Clinical Director of ServCor Hospital.
3. PhD in Cardiovascular Surgery; Director of Cardiovascular Devices Division and Chair of Medical Devices and Medical Engineering - University of Ottawa.
4. PhD in Cardiovascular Surgery; Director of Heart Transplantation Department and Circulatory Support of INCOR – USP.
5. PhD in Cardiovascular Surgery; Coordinator of Postgraduation in Cardiovascular Surgery of São Francisco de Assis Foundation.

Correspondence address: Denoel Marcelino de Oliveira
Av. Das Américas, 4200 bloco 8-A grupo 208 - Edifício Genéve Centro Empresarial Barra Shopping – Barra da Tijuca – Rio de Janeiro, RJ, Brasil – CEP: 22640-102
E-mail: drdenoel@hotmail.com

OLIVEIRA, DM ET AL - Effects of n-acetylcysteine on ischemic preconditioning. Study in isolated rat hearts

Rev Bras Cir Cardiovasc 2009; 24(1): 23-30

CO_2). GI: Controle (n=6); GII: 20 min. isquemia (n=6); GIII: PC (n=6); GIV 50 μg/ml/min NAC antes do PC (n =6); GV: 100 μg/ml/min NAC antes do PC (n=6). Todos os parâmetros foram mensurados após 15 minutos de estabilização (T0) e T3, T5, T10, T15, T20, T25 e T30 minutos de reperfusão. Significância estatística foi considerada quando *P*<0,05.

Resultados: Foram observadas alterações na FC comparando GI com GII em T20 e T25 e comparando GI com GIII e GIV com GV em T10 e T20 (*P*<0,05). FLC foi diferente comparando GI com GII em T3 e T5, GI com GIV em T10 e GI com GV em T10 e T25 (*P*<0,05). dP/dt foi semelhante comparando GIII com GI e GV. GIII apresentou maior dP/dt que GIV, mas sem diferença estatística (*P*>0,05). dP/dt foi maior no GV comparado com GIV, mas com diferença estatisticamente significativa somente em T30.

Conclusão: Os corações precondicionados tiveram melhor dP/dt, sendo alteradas pelo uso de NAC no GIV e não alteradas no GV.

Descritores: Precondicionamento isquêmico. Infarto do miocárdio. Contração miocárdica. Radicais livres. Experimentação animal.

INTRODUCTION

Myocardial protection during heart surgery has been the focus of basic and clinical research in the last 50 years [1]. In 1955, Lewis was the first to perform in human intracardiac surgery using hypothermia. Melrose proposed the use of cardiac arrest using an infusion of potassium in the ascending aorta. Since then, numerous tactics and techniques of myocardial protection have been developed.

Murry et al. [2] described the mechanism of ischemic preconditioning (IP) by showing that short episodes of ischemia and reperfusion before a prolonged ischemic event would reduce the size of infarction and improved cardiac function. In experimental studies, this mechanism is considered the most powerful natural way of cardioprotection [3]. Studies with IP in heart surgery have shown conflicting results, but the majority confirmed that the IP is an effective adjunct in myocardial protection [3-5].

Clinical studies in cardiology and heart surgery showed tendency to cardioprotection by using adenosine, but the effects are not as obvious as those observed in experimental researches [3,4]. Morris and Yellon [6] emphasized the existence of a threshold of stimulation, with the need of activation not only of adenosine receptors, but also of bradykinin and opioids. Researches and investigations on the molecular mechanisms of IP may prove applicable techniques or drugs, with clinical benefit in heart surgery.

The biochemical mechanisms of stimulation of IP involve a variety of G receptors of cytoplasmic membrane leading to the translocation of protein kinase C (PKC) from the cytoplasm to the sarcolemma [3]. Nakano et al. [7] showed that the translocation of PKC is related to activation of mitoK$^+_{ATP}$ channel through the stimulation of kinases positioned just below the mitogenic-activated protein kinase p38 (MAPK). The regulation of mitochondrial volume and transport of electrons are the main mechanisms responsible for maintaining its function and are linked to the opening of mitoK$^+_{ATP}$ channels [8].

The release of oxygen free radicals (OFR) is a cause of myocardial lesion during post-ischemia reperfusion. Paradoxically, Pain et al. [9] and Forbes et al. [10] showed that the release of reactive oxygen species (ROS) participates in the protective mechanism of the IP by opening of mitoK$^+_{ATP}$ channels and activation of kinases.

N-acetylcysteine is classified as an antioxidant, but has broad clinical utility as a mucolitic agent, as antidote for acetaminophen poisoning, prevention of renal dysfunction by use of radiocontrasts and potentiator of hemodynamic effects of nitroglycerin. Sochman [11] proposed four possible antioxidant mechanisms for the N-acetylcysteine: (1) direct connection, and free radicals elimination by the Redox (cysteine-cysteine) reaction (2) modulation of injured endothelium, (3) production of disulfito during reaction of sulphidric group of N-acetylcysteine with the enzyme membrane's group and as (4) precursor of glutathione.

Forbes et al. [10] showed that N-acetylcysteine blocked the improvement of post-ischemic contractile function after induction of IP with diazoxide - an opener of K$^+_{ATP}$ channels. Chen et al. [12] noted that N-acetylcysteine blocked the loss of glutathione and the protective effect of IP, but its protocol with four cycles of 5 minutes of ischemia and reperfusion resulted in myocardial stunning before the sustained period of ischemia. Pain et al. [9] proposed that the OFR had a paradoxical role in the protection provided by IP, encouraging us to study the effects of antioxidant N-acetylcysteine on the mechanism of IP in isolated rat hearts according Langendorff model.

N-acetylcysteine has wide clinical use, and is used in many patients undergoing heart surgery. The study of Forbes et al. [10] and Chen et al. [12] showed blocking of the protective effects of IP by N-acetylcysteine, but Forbes et al. [10] induced myocardial lesion with excessive cycles

OLIVEIRA, DM ET AL - Effects of n-acetylcysteine on ischemic preconditioning. Study in isolated rat hearts

Rev Bras Cir Cardiovasc 2009; 24(1): 23-30

of ischemia and reperfusion and Chen et al. [12] did not mechanically stimulated the IP, but using diazoxide. Thus, this study aims to study the effects of N-acetylcysteine on the heart rate (HR), coronary flow (CF) and myocardial contractility (dP/dt) in isolated rat hearts undergone only one cycle of mechanic IP.

METHODS

The animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1985). We followed the ethical principles for the use of laboratory animals established by the Brazilian College of Animal Experimentation (COBEA) and Brazilian Society of Laboratory Animals Science.

Thirty Wistar rat hearts were studied. All animals were adults, with weight ranging from 220 to 330 grams. The animals were supplied by the bioterium of the São Francisco de Assis Cardiovascular Foundation. The study was previously approved by the Foundation's Research Ethics Committee.

The animals underwent inhalation anesthesia with sulfuric ether in closed bell-shaped. The large vessels were well exposed by an anterior thoracotomy and anticoagulation was performed with injection of heparin sodium 500 IU. The ascending aorta was dissected and isolated using 2-00 cotton thread, and then cannulated with 20G catheter, taking care to preserve the aortic valve and coronary ostia. The left ventricle drainage was performed by inserting a 18G side hole catheter, from the left appendage to the left ventricle apex passing through the mitral valve. Special care was taken to avoid lesion in the coronary vessels, as described by Oliveira et al. [13].

After 15 minutes of stabilization, a water-filled latex balloon was positioned inside the left ventricle through the left atriotomy, by maintaining a diastolic pressure of 8 ± 2 mmHg. Global ischemia was induced by interrupting the perfusate flow and maintaining the balloon deflated. The latex balloon was connected by a flexible extension to a pressure transducer. Systolic and diastolic pressures, heart rate (HR) and electrocardiogram (ECG) were recorded in biomonitor and printer (DH Mod 073, BESE - Bioengenharia, Belo Horizonte, MG.). The coronary flow was measured by collection of effluent for a period of one minute (Figure 1).

The results were expressed as control values percentage (mean $\pm$ standard deviation). The Student's t test was used to assess statistical significance of differences between paired observations. The difference was considered statistically significant when $P <0.05$.

The comparison of means between groups was performed using Student's t test and Bartlett's test to assess homogeneity of va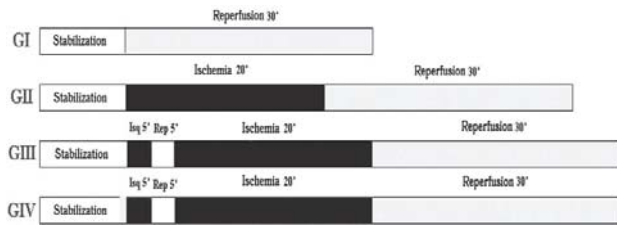riance. ANOVA was applied when there was homogeneity of variance and non-parametric Kruskal-Wallis test was used when the variables were not homogeneous in the Bartlett's test.

Protocols of the groups studied

The Control Group (GI) consisted of six hearts prepared by the method described and perfused using Krebs-Henseleit solution at 37°C for 15 minutes for stabilization. After this period, the latex balloon was inserted into the left ventricle and the heart perfused for 30 minutes. Heart rate, ventricular pressure and coronary flow were measured in all groups at 3, 5, 10, 15, 20, 25 and 30 minutes of reperfusion. The ischemia group (GII) consisted of six hearts undergone 20 minutes of global ischemia after the stabilization period and all parameters were measured during reperfusion, as previously described. The preconditioning Group (GIII) consisted of six hearts udergone five minutes of global ischemia, followed by five minutes of reperfusion prior to 20-minutes ischemia. All parameters were measured in periods of reperfusion previously described. Group IV (GIV) received infusion of 50 µg/ml/min and the Group V (GV) 100µg/ml/min of N-acetylcysteine for one minute at the infusion line next to the heart, prior to the preconditioning protocol (Figure 2).
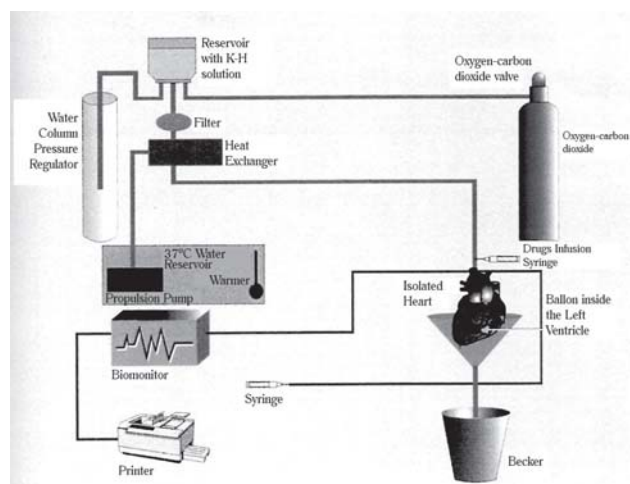
1 - Schematic drawing of the disposable system for study of isolated heart by the Langendorf method. The Krebs-Hensleit infusion solution is pressurized by the oxygen-carbon dioxide debubbling in the reservoir. Under a pressure maintained at 100 cm of water, the solution perfuses the isolated heart by a temperature of $37\pm0.5°C$ after passing through the heat exchanger. A latex balloon is positioned within the left ventricle, with diastolic pressure maintained at 8 ± 2 mmHg for monitoring of contractility and recording in printer. A route of drugs infusion is positioned anterior to the isolated heart

OLIVEIRA, DM ET AL - Effects of n-acetylcysteine on ischemic preconditioning. Study in isolated rat hearts

Rev Bras Cir Cardiovasc 2009; 24(1): 23-30

2 - Scheme of perfusion protocols with their temporal effects. Isc. - ischemia, Rep - reperfusion

RESULTS

Weights of animals and hearts

The weight of animals ranged from 220.0 to 3.300 grams. The mean weight of the hearts can be seen in Table 1, which is statistically higher in group III, compared to other groups (P <0.05).

Heart Rate variation

Heart rate in Group I (control) ranged with values lower than the initial time, but without statistical differences between periods when compared to T_0 ($P>0.05$). In Group II, the mean heart rate showed only statistically significant decrease in T_{20} ($P<0.05$). In Group III (Ischemic Preconditioning), there was statistical difference in T_{10} and T_{20} when compared to T_0 ($P<0.05$). Group IV showed significant decrease in T_{10}, T_{15} and T_{20} if compared to T_0. In Group V, the lowest value presented statistical difference in T_{30} ($P<0.05$).

Comparing the heart rate of Group I with Group II, there was statistical difference only at T_{20} and T_{25} ($P<0.05$). Groups III, IV and V - when compared with Group I - showed statistical difference in T_{10} and T_{20} ($P<0.05$) (Figure 3).
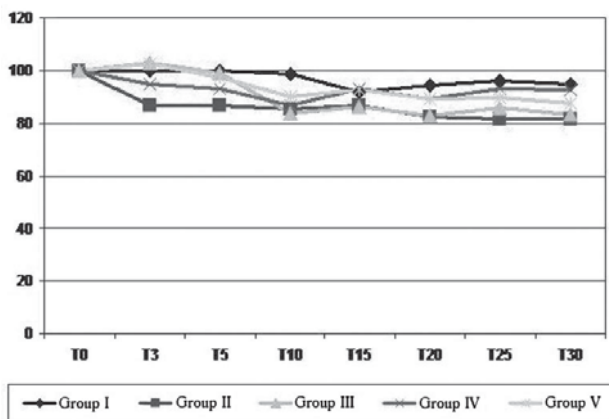
3 - Percentage variation in heart rate in beats per minute, within the periods studied in each group

Coronary flow

The coronary flow in Group I remained stable during the study, with statistical difference only at T_5 ($P<0.05$). In Group II (20 minutes of ischemia), coronary flow values decreased during periods with statistically significant difference in all periods when compared to T_0 ($P<0.05$). In Group III (ischemic preconditioning), statistical differences were noted only in T_{25} when compared to T_0 ($P<0.05$). The coronary flow decreased gradually in Group IV, showing statistically significant differences in all periods, when compared to T_0 ($P<0.05$). In Group V, there was statistical difference in the T_{25} and T_{30}, if compared with T_0 ($P<0.05$).

Comparing the coronary flow of Group I with Group II, there were statistical differences only at T_3 and T_5, with $P<0.05$. There were no differences between Groups I and III. The coronary flow of the Group I was statistically different from Group IV at T_{10}. Group I - when compared to Group V - showed differences in T_{10} and T_{25}. Other comparisons between groups showed no statistical differences in coronary flow (Figure 4).
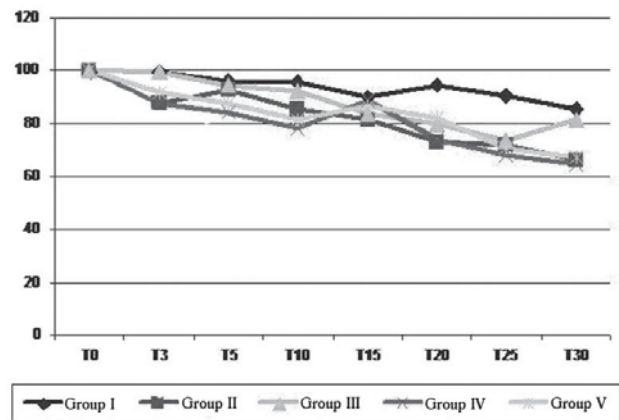

Fig.4 - Percentage variation in coronary flow in milliliters per minute, within the periods studied in each group

Myocardial contractility (dP/dt)

The myocardial contractility (dP/dt) in Group I was statistically different only in T_{30}, if compared to T_0 ($P<0.05$). The dP/dt in Groups II and IV was statistically reduced in all periods when compared to T_0 ($P <0.05$). The dP/dt in Group III and V showed no statistical differences between the periods studied.

OLIVEIRA, DM ET AL - Effects of n-acetylcysteine on ischemic
preconditioning. Study in isolated rat hearts

Rev Bras Cir Cardiovasc 2009; 24(1): 23-30

Myocardial contractility was statistically depressed during all periods, by comparing the Group I and Group III with Group II (P<0.05). The Group of ischemic preconditioning (GIII) presented dP/dt statistically similar to control group (GI) and the groups treated with N-acetylcysteine (GIV and GV), *P>0.05*. Groups GIII, GIV and GV presented contractility statistically superior to the GII in all periods studied (*P<0.05*). Group IV (50µg/ml/min N-acetylcysteine and IP) showed dP/dt statistically depressed (P<0.05) in T_{10}, T_{15} and T_{30}, compared to the control group and higher than Group II at T_3 and T_5. Group V (100µg/ml/min N-acetylcysteine and IP) presented greater myocardial contractility than Group II, with statistical difference in all periods and was depressed at times T_{10} and T_{15} compared to the control group (Figure 5) (Table 1).
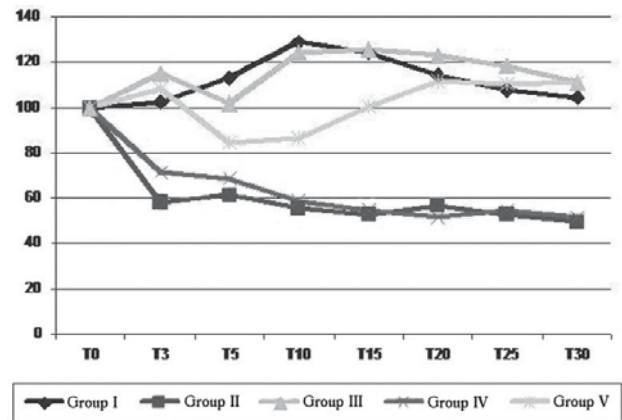

Fig.5 - Percentage variation in myocardial contractility (dP/dT) in millimeters of mercury per second, within the periods studied in each group

Table 1. Table showing the statistical comparison of the percentage difference of myocardial contractility (dP/dT) among the studied groups.

Comparison among groups	Statistical comparison of myocardial contractility among the studied groups in each period							
	T_0	T_3	T_5	T_{10}	T_{15}	T_{20}	T_{25}	T_{30}
	% Dif							
GI - GII	P	43.7	51.09	73.5	71.1	57.1	60.3	54.9
		0.009	0.01	0.004	0.004	0.01	0.01	0.004
GI - GIII	0	15.5	31.7	39.2	34.7	24.4	27.7	24.1
		0.36	0.2	0.11	0.13	0.27	0.26	0.28
GI - GIV	0	20.5	35.9	49.6	46.4	37.2	38.1	33.7
		0.12	0.08	0.019	0.019	0.07	0.08	0.04
GI - GV	0	13.9	34.0	47.8	40.7	28.6	29.4	18,9
		0.27	0.1	0.01	0.03	0.17	0.19	0.21
GII - GIII	0	-56.5	-40.4	-68.8	-72.7	-69.4	-65.7	-61.4
		0.004	0.006	0.004	0.004	0.004	0.004	0.004
GII - GIV	0	-34.7	-23.9	-36.0	-37.1	-29.7	-33.7	-31.7
		0.004	0.02	0.07	0.07	0.18	0.06	0.07
GII - GV	0	-39.7	-23.8	-34.3	-40.6	-38.0	-41.6	-47.9
		0.001	0.02	0.01	0.01	0.04	0.01	0.01
GIII - GIV	0	-3.0	4.6	17.3	11.8	8.4	3.2	-10.5
		0.6	0.81	0.4	0.64	0.48	0.77	0.7
GIII - GV	0	-3.0	4.6	17.3	11.8	8.4	3.2	-10.5
		0.6	0.81	0.41	0.64	0.48	0.77	0.72
GIV - GV	0	-26.5	-7.5	-7.0	-22.9	-34.6	-34.8	-59.2
		0.07	0.59	0.25	0.08	0.07	0.07	0.009

OLIVEIRA, DM ET AL - Effects of n-acetylcysteine on ischemic preconditioning. Study in isolated rat hearts

Rev Bras Cir Cardiovasc 2009; 24(1): 23-30

DISCUSSION

Cardioprotection during heart surgery remains a subject that requires many studies [1]. Since the description of IP by Murry et al. [2] in 1986, many researchers have attempt to disclose the mechanisms and directions of this phenomenon [3-10]. If the IP is considered the most powerful known natural form of cardioprotection in experimental research [3], why not use this powerful protective mechanism in heart surgery? Some clinical studies showed a protective effect associated with the stimulation of forms of IP by using adenosine and sevoflurane during heart surgery [4,5]. However, the clarifying of the whole mechanism may result in a new mediator, channel and/or an end effector that can clinically stimulate the powerful cardioprotective effects of IP.

In 1997, Garlid et al. [8] described the mitoK+$_{ATP}$ channel as the end effector of IP and, in 2000, Pain et al. [9] found that the mitoK+$_{ATP}$ channel is a trigger for IP by generating the OFR. Jeroudi et al. [14] have established that high levels of OFR were damaging to the myocardial tissue. Otherwise, moderate doses of H_2O_2 e O_2^- have shown to stimulate cardioprotective effect similar to that observed in IP, as shown by Baines et al. [15]. In this study, the protective mechanism of IP was reduced by administration of 50µg/ml/min of the N-acetylcysteine antioxidant, as observed by depression of dP/dt of the Group IV, when compared to Group III.

Chen et al. [12] showed that N-acetylcysteine blocks the effects of IP. However, its preconditioning protocol with four cycles of 5 minutes of ischemia followed by 5 minutes of reperfusion resulted in myocardial stunning, reducing the left ventricle contractility to 72% from the initial value at the end of the fourth cycle of reperfusion. In this study, only one cycle of IP showed to offer cardioprotection by comparing the dP/dt of Groups II and III with the control group (GI).

The protective action of N-acetylcysteine on the myocyte in the reperfusion injury is due to its antioxidant effects and capture of oxygen free radicals and is used clinically in unstable coronary events, such as acute myocardial infarction [11]. Hsu et al. [16] reported that N-acetylcysteine increases production of nitric oxide (NO) in experimental studies and Delgado et al. [17] showed endothelium protection stimulated by the use of N-acetylcysteine. This confirms the protection obtained by use of N-acetylcysteine in the reperfusion injury and release of NO with potentiation of the nitroglycerin effects observed in previous studies [11]. Singh et al. [18] showed that reducing agents - such as glutathione - can stimulate the storage and transport of NO. Increased production of NO by using N-acetylcysteine may explain why the Group V showed no significant change of dP/dt, despite its antioxidant action.

Although the NO has been related to triggering and end effector of late preconditioning, evidence of its participation in the classic preconditioning are limited - or controversial - as reported by Nakano et al. [19]. Bell and Yellon [20] showed that in classic preconditioning, NO seems to low the threshold for the such protection, even if it is not a direct trigger of classic IP.

In 1993, Kuzuya et al. [21] reported a late phase of myocardial protection, less intense but more prolonged that lasted 72 hours. The authors attributed this effect to increasing of antioxidant enzymes such as MnSOD. This late phase was named Second Window of Protection (SWP). The SWP provides protection by synthesis, modification, and compartmentalization of proteins.

Hoshida et al. [22] were the first to show that the increase of the system of antioxidant enzymes (such as the Superoxide Dismutase (MnSOD)) could stimulate late protective ischemic effects in dog hearts. The two main antioxidant enzymes systems of the heart are the Superoxide Dismutase (SOD) and Glutathione Peroxidase, both stimulated by the use of N-acetylcysteine, as previously described.

Since 1994, Baxter and Yellon [23] described the stimulation of Adenosine 1 receptors as the main trigger of SWP, and in 1999, concluded that the K+$_{ATP}$ channel mediated the cardioprotective effect of activation of Adenosine A1 receptors. This fact supports the theory that the release of OFR by activation of ATP-sensitive K+ channel is a cardioprotective process that begins after 12 hours from stimulation and can last up to 72 hours.

The results of myocardial contractility in Group IV support the hypothesis that OFR is important in initial stimulation of IP classic protection. The period of classic ischemic preconditioning protection is very short, lasting only one to two hours in anesthetized animals and is lost around two to four hours in studies with conscious rabbits [24]. Takano et al. [25] showed that SWP of IP protects both against myocardial stunning or infarction, and can be stimulated by the use of NO donors.

If we are attempting to find drugs that may protect the heart during surgical anoxia, such drugs must also incorporate the second phase of the mechanism, which lasts between 12 and 72 hours. The SWP is a strong adjunct, because it may also protect against ischemic events during postoperative period.

The use of higher dose of N-acetylcysteine in this study, has shown it did not abolish the classic phase of Ischemic Preconditioning, probably due to its effect on the increase of NO, which should low the threshold of IP protection. This effect was noted although the NO is not the direct trigger of early preconditioning [20]. However, its effects

OLIVEIRA, DM ET AL - Effects of n-acetylcysteine on ischemic preconditioning. Study in isolated rat hearts

Rev Bras Cir Cardiovasc 2009; 24(1): 23-30

on SWP can be enormous, not only due to release of NO, but also by the increase of antioxidant enzymes [25].

CONCLUSION

The use of only one cycle of ischemia and reperfusion is effective in triggering myocardial protection – IP - in this experimental model.

50µg/ml/min administration of N-acetylcysteine significantly inhibited the ischemic preconditioning mechanism stimulated by a cycle of 5 minutes of ischemia and reperfusion.

100µg/ml/min administration of N-acetylcysteine did not alter significantly the stimulation of IP. Possibly, the double dose of N-acetylcysteine offsets the inhibition of IP antioxidant, by the adjunct effects of endothelial protection, releasing of NO with decrease of the PC threshold and stimulus of antioxidant enzymes.

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OLIVEIRA, DM ET AL - Effects of n-acetylcysteine on ischemic preconditioning. Study in isolated rat hearts

Rev Bras Cir Cardiovasc 2009; 24(1): 23-30

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