

## THE PROTECTIVE EFFECTS OF HIGH DOSE ASCORBIC ACID AND DILTIAZEM ON MYOCARDIAL ISCHAEMIA-REPERFUSION INJURY

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### Summary

In this study, we aimed to compare the myocardial protective effects of high dose ascorbic acid with the effects obtained by adding diltiazem to high dose ascorbic acid. We studied 30 elective cardiac surgery patients prospectively. In ascorbic acid group (group AA), ascorbic acid was given after induction and just before aortic declamping, 50 mg.kg<sup>-1</sup> each time. In ascorbic acid + diltiazem group (group AA + D), diltiazem was added to ascorbic acid (0.3 mg.kg<sup>-1</sup>, i.v. after induction and then 2 µg.kg<sup>-1</sup> min<sup>-1</sup> i.v. infusion until declamping). Group C was the control group. There was no significant difference between groups in terms of cardiac enzyme levels. After declamping, the arterial and coronary sinus malondialdehyde levels, measured as a marker of lipid peroxidation, were increased significantly in the group C while remained stable in the other two groups. Ventricular fibrillation (VF) after declamping was positive in 3, 1 and 6 patients in the groups AA, AA + D and C respectively. In this study, we observed the prevention of lipid peroxidation in the group AA

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and group AA + D. The only positive result obtained by addition of diltiazem to high dose ascorbic acid was the decrease in the frequency of VF after declamping. We concluded that the prevention of lipid peroxidation in the groups AA and AA + D provided no measurable protection over myocardial ischaemia-reperfusion injury.

**Key words:** Ascorbic acid, diltiazem, myocardium, reperfusion injury, malondialdehyde.

## Introduction

Cardiopulmonary bypass (CPB) is associated with the risk of ischaemia-reperfusion injury. While ischaemia produces deleterious effects on the myocardium by means of diminished oxygen supply and accumulation of metabolic byproducts<sup>1</sup>, restoration of blood flow during cardioplegia administration and after declamping of the aorta leads to reperfusion injury<sup>2</sup>. There is a considerable evidence implicating oxygen-derived free radicals (ODFR) and intracellular calcium ( $\text{Ca}^{+2}$ ) overload as two of the important mediators of ischaemia-reperfusion injury<sup>3,4</sup>.

It is hypothesized that large quantity of ODFR overwhelming body's endogenous antioxidant defenses leads to peroxidation of lipid membranes and loss of membrane integrity<sup>5</sup>. As a result massive influx of  $\text{Ca}^{+2}$  can occur and this  $\text{Ca}^{+2}$  redistribution may amplify the damage produced by the ODFR<sup>6</sup>. Recognition of these proposed mechanisms has led to the development of methods to prevent the formation of ODFR and lipid peroxidation<sup>7-10</sup> and to limit  $\text{Ca}^{+2}$  influx by the use of drugs that block  $\text{Ca}^{+2}$  entry into cells<sup>11,14</sup>.

Our study was designed to compare the protective effects of high dose ascorbic acid on ischaemia-reperfusion injury with the effects obtained by adding a  $\text{Ca}^{+2}$  channel blocker, diltiazem to high dose ascorbic acid aiming to prevent the two important mediators of injury simultaneously.

## Materials and methods

We studied 30 patients (24 male, 6 female) undergoing elective coronary artery bypass grafting (CABG). The mean age was  $63.8 \pm 8.5$  years (range: 44 to 75 years). The study was approved by the University Ethics Committee and informed consent was signed by each patient. Exclusion criteria were recent myocardial infarction (within last 6 weeks), angina resistant to medical treatment, preoperative use of  $\text{Ca}^{+2}$  channel blockers or medications with known antioxidant activity, poor left ventricular function (ejection fraction  $< 40\%$ ) and additional organ dysfunction. The patients were randomly divided into three groups, 10 patients each. In ascorbic acid group (group AA), ascorbic acid was given after induction and just before aortic declamping,  $50 \text{ mg.kg}^{-1}$  each time. In ascorbic acid + diltiazem group (group AA + D), in addition to the same dose of ascorbic acid, diltiazem  $0.3 \text{ mg.kg}^{-1}$  (i.v.) was given after induction and continued as  $2 \text{ } \mu\text{g.kg}^{-1}.\text{min}^{-1}$  (i.v.) infusion until declamping. Group C was the control group.

The patients were premedicated with diazepam 10 mg and morphine  $0.1 \text{ mg.kg}^{-1}$ , both given 1 hr before operation. ECG monitoring (leads II and V5) was started upon arrival to the operating theater. A radial artery cannula was placed before induction for hemodynamic monitoring and blood sample collection. Anaesthesia was induced with fentanyl  $10 \text{ } \mu\text{g.kg}^{-1}$ , midazolam  $0.15 \text{ mg.kg}^{-1}$  and pancuronium  $0.1 \text{ mg.kg}^{-1}$ , followed by a continuous infusion of fentanyl  $5\text{--}10 \text{ } \mu\text{g.kg}^{-1}.\text{h}^{-1}$  and enflurane inhalation up to 1.5% in the inspiratory gas flow. During CPB, fentanyl infusion was decreased to  $5 \text{ } \mu\text{g.kg}^{-1}.\text{h}^{-1}$  and continued till the end of the operation. After induction of anaesthesia, the trachea was intubated and the lungs were mechanically ventilated with a mixture of oxygen and air ( $\text{FiO}_2 = 0.5$ ). Normocapnia was maintained and assessed continuously by end-tidal carbon dioxide concentration and intermittently by arterial blood-gas analyses. After induction, central venous and pulmonary artery catheters were inserted and pressures were monitored continuously during the operation. Throughout anaesthesia and surgery, pancuronium

was given for neuromuscular block as needed.

### *Cardiopulmonary Bypass*

The aorta and right atrium were cannulated for CPB which was conducted with a semi-occlusive roller pump to produce a non-pulsatile flow, and with a membrane oxygenator. The CPB circuit was primed with 1000 ml of 5% dextrose in lactated Ringer's solution, 1000 ml of 0.9% saline and heparin 2500 u. Systemic anticoagulation was achieved with heparin  $400 \text{ u.kg}^{-1}$  and with additional doses to maintain the activated coagulation time (ACT) longer than 480 sec during CPB. Pump flow was adjusted to  $2.4 \text{ L.min}^{-1}.\text{m}^{-2}$  and reduced to  $1.5\text{--}1.8 \text{ L.min}^{-1}.\text{m}^{-2}$  during hypothermia (rectal temperature  $28^{\circ}\text{C}$ ). For myocardial protection during cross-clamping, topical myocardial cooling and multidose antegrade cold blood potassium cardioplegia solution were used (after an induction dose of  $5 \text{ ml.kg}^{-1}$ , the same dose was repeated at every 20 minutes). A terminal infusion of  $10 \text{ ml.kg}^{-1}$  normothermic cardioplegia solution (hot shot) was given into the aortic root before cross-clamp removal. Mean arterial pressure was kept between 50-60 mmHg. Normocapnia was maintained according to the  $\alpha$ -stat principle, that is alveolar carbon dioxide tension values were not corrected to the actual temperature of the patient. After CPB, protamine sulphate was given to antagonize heparin in a 1:1 ratio with the initial dose of heparin. Additional protamine was administered if ACT was  $> 140$  sec. During the post-CPB period, the residual blood remaining in the extracorporeal circuit was collected and transfused into the patient. During the operation, medications with known antioxidant properties were avoided.

### *Sample Collection and Measurements*

Before the onset of CPB, coronary sinus (CS) catheter was introduced. Blood samples for measurement of malondialdehyde (MDA) levels were collected from the radial artery and CS catheters (Art-MDA and CS-MDA). Samples were taken: (1) before induction of anaesthesia (Art-MDA<sub>0</sub>), (2) after CS

catheterization (Art-MDA<sub>1</sub> and CS-MDA<sub>1</sub>), (3) after declamping (Art-MDA<sub>2</sub> and CS-MDA<sub>2</sub>), and (4) after protamine administration (Art-MDA<sub>3</sub>) and plasma was separated from the cellular component within an hour. The plasma samples were stored at - 20°C by addition of glutathione and EDTA. MDA levels as a marker of lipid peroxidation were measured by thiobarbituric acid reaction using a spectrophotometric technique modified from Yagi<sup>15</sup> and Satoh<sup>16</sup>. 1,1,3,3-tetraethoxypropane was used as a standard and the results were expressed as nmol.ml<sup>-1</sup>.

Before induction and after declamping, venous blood was drawn for plasma ascorbic acid level measurements which were carried out by spectrophotometric method (color changing red when mixed with dinitrophenylhydrazine). Plasma creatine phosphokinase (CPK), MB isoenzyme of creatine kinase (CK-MB) and lactate dehydrogenase (LDH) levels were measured preoperatively and at the second postoperative hour (Hitachi 704 automatic analyzer, Tokyo, Japan) which was the nearest time where the enzymes reached the peak levels<sup>9</sup>.

Hemodynamic variables were measured at the following stages: (1) before incision, (2) before the onset of CPB, (3) after protamine administration, and (4) at the second postoperative hour. The measurements included heart rate, mean arterial pressure, mean pulmonary artery pressure, central venous pressure, pulmonary capillary wedge pressure and cardiac output (CO). CO was determined by the thermodilution method. Ten milliliters of 0.9% saline at room temperature was injected at end expiration. The mean value derived from three well-formed thermodilution curves was calculated. From these variables, cardiac index (CI) and systemic and pulmonary vascular resistances were calculated according to standard formulae. During the spontaneous recovery of myocardial activity after declamping, the occurrence of ventricular fibrillation (VF) was recorded.

### *Statistical Analysis*

Data are expressed as mean  $\pm$  standard deviation. Intra-

group comparisons were performed by ANOVA with repeated measures design followed by the Newman Keuls test for post hoc comparison. Significance of differences among the different groups was tested by one-way ANOVA. For the ANOVA resulting with significant F values, comparison between groups was performed using least significant difference (LSD) test. Fischer's exact test was used to compare the frequency of VF between groups since catagorical data was involved. The level of significance was  $P < 0.05$  unless otherwise indicated.

## Results

Details of patients and surgery are shown in Table 1. The groups were comparable in terms of patient characteristics and operative data. There was no mortality during the study procedure.

Table 1:  
Preoperative patient characteristics and operative data (mean  $\pm$  SD).

	Group C (Control)	Group AA (Ascorbic acid)	Group AA + D (Ascorbic acid + diltiazem)
n	10	10	10
Age(yr)	64(45-73)	67(54-73)	61(44-73)
BSA(m <sup>2</sup> )	1.8 $\pm$ 0.2	1.8 $\pm$ 0.2	1.8 $\pm$ 0.1
EF(%)	46 $\pm$ 5	49 $\pm$ 5	47 $\pm$ 4
Duration of operation (min)	358 $\pm$ 56	351 $\pm$ 56	315 $\pm$ 57
Duration of CPB (min)	127 $\pm$ 39	133 $\pm$ 32	128 $\pm$ 33
Cross-clamping time (min)	71 $\pm$ 27	85 $\pm$ 28	81 $\pm$ 23
Grafts/patient	3.7 $\pm$ 0.4	3.8 $\pm$ 0.4	3.5 $\pm$ 0.8

BSA = Basal surface area; EF = ejection fraction; CPB = cardiopulmonary bypass.

Plasma ascorbic acid levels were increased about ten-fold in the groups AA and AA + D (Table 2) ( $P < 0.001$ ). The

preoperative and postoperative cardiac enzyme levels are summarized in Table 2. The cardiac enzyme levels (CPK, CK-MB, LDH) were increased significantly in all groups at the second postoperative hour when compared with the preoperative values ( $p < 0.001$ ). All three enzyme levels tended to be lower in the group AA + D, without the difference reaching statistical significance.

Table 2:  
Preoperative and postoperative plasma ascorbic acid and  
cardiac enzyme levels (mean  $\pm$  SD).

	Group C (Control)	Group AA (Ascorbic acid)	Group AA + D (Ascorbic acid + diltiazem)
Preop AA (mg.dl <sup>-1</sup> )	1.09 $\pm$ 0.51	1.11 $\pm$ 0.53	1.39 $\pm$ 1.02
Postop AA	1.19 $\pm$ 0.74	11.61 $\pm$ 1.37**	9.60 $\pm$ 3.59**
Preop CPK (UL <sup>-1</sup> )	83.5 $\pm$ 48.5	86.3 $\pm$ 52.4	79.4 $\pm$ 47.1
Postop CPK	534.2 $\pm$ 221.0**	526.5 $\pm$ 194.0**	407.5 $\pm$ 171.1**
Preop CK-MB(UL <sup>-1</sup> )	17.9 $\pm$ 7.3	17.1 $\pm$ 6.0	16.7 $\pm$ 4.1
Postop CK-MB	74.1 $\pm$ 21.0**	68.6 $\pm$ 16.3**	62.0 $\pm$ 8.3**
Preop LDH (UL <sup>-1</sup> )	486.2 $\pm$ 114.1	498.9 $\pm$ 182.7	418.2 $\pm$ 124.3
Postop LDH	1013.0 $\pm$ 306.9**	1037.2 $\pm$ 256.6**	992.1 $\pm$ 225.1**

AA = Ascorbic acid; CPK = creatine phosphokinase; CK-MB = MB isoenzyme of creatine kinase; LDH = lactate dehydrogenase. \*\* $P < 0.001$  vs preoperative level.

The MDA levels obtained from arterial and coronary sinus blood were shown in Table 3. There was no significant change in the MDA levels after induction and after coronary sinus catheterization. After declamping, both the arterial and coronary sinus MDA levels were increased significantly in the group C while there was no significant change in the other two groups. The arterial and CS-MDA levels after declamping in the groups AA and AA + D were significantly lower than the level in the group C. The simultaneously obtained arterial and coronary sinus MDA levels were comparable in all groups.

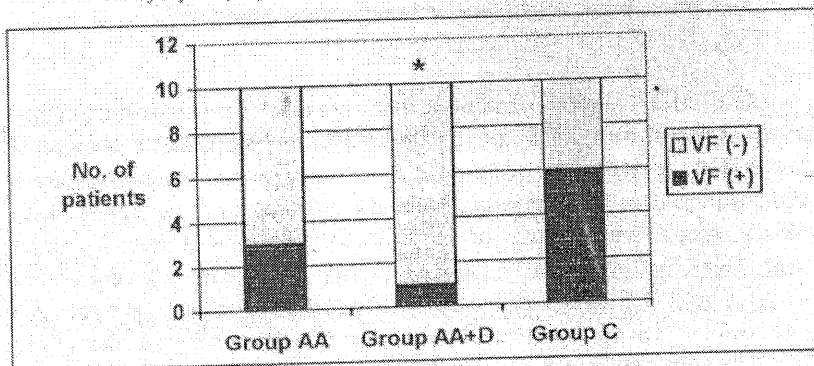
Table 3:  
MDA levels obtained from arterial and coronary sinus blood (mean  $\pm$  SD)

	Group C (Control)	Group AA (Ascorbic acid)	Group AA + D (Ascorbic acid + diltiazem)
Art-MDA <sub>0</sub> (nmol.ml <sup>-1</sup> )	2.00 $\pm$ 0.34	2.56 $\pm$ 0.92	2.36 $\pm$ 0.55
Art-MDA <sub>1</sub>	1.95 $\pm$ 0.62	2.55 $\pm$ 0.94	2.35 $\pm$ 0.63
Art-MDA <sub>2</sub>	3.44 $\pm$ 0.88 <sup>#</sup>	2.32 $\pm$ 0.74 <sup>*</sup>	2.55 $\pm$ 0.62 <sup>*</sup>
Art-MDA <sub>3</sub>	3.64 $\pm$ 0.81 <sup>#</sup>	2.73 $\pm$ 0.61 <sup>*</sup>	2.72 $\pm$ 0.68 <sup>*</sup>
CS-MDA <sub>1</sub>	2.35 $\pm$ 0.89	2.72 $\pm$ 0.68	2.39 $\pm$ 0.70
CS-MDA <sub>2</sub>	3.67 $\pm$ 0.72 <sup>**</sup>	2.61 $\pm$ 0.91 <sup>*</sup>	2.14 $\pm$ 0.45 <sup>*</sup>

Art-MDA<sub>0</sub> = Arterial MDA before induction; Art-MDA<sub>1</sub> = arterial MDA after CS catheterization; Art-MDA<sub>2</sub> = arterial MDA after declamping; Art-MDA<sub>3</sub> = arterial MDA after protamine administration; CS-MDA<sub>1</sub> = coronary sinus MDA after CS catheterization; CS-MDA<sub>2</sub> = coronary sinus MDA after declamping. <sup>\*</sup> P < 0.05 vs Art-MDA<sub>0</sub>; <sup>#</sup> P < 0.05 vs Art-MDA<sub>1</sub>; <sup>\*</sup> P < 0.05 vs group C; <sup>\*\*</sup> P < 0.001 vs CS-MDA<sub>1</sub>.

VF after declamping was positive in 3, 1 and 6 patients in the groups AA, AA + D and C respectively (Figure 1). The difference between the groups AA + D and C was significant. The hemodynamic measurements showed similar fluctuations in all groups.

Figure 1:  
The frequency of ventricular fibrillation (VF) after declamping.



AA = Ascorbic acid; AA + D = ascorbic acid + diltiazem;  
C = control. <sup>\*</sup> P < 0.05 vs group C.



## Discussion

During the period of ischaemia-reperfusion, the large amounts of ODFR produced exceeds the capacity of body's defense. Therefore, supplementation with antioxidants have been the subject of many studies<sup>9,10,17</sup>.

The main antioxidant action of ascorbic acid is the rapid reduction of ODFR. Also it takes part in conversion of tocopheryl radical, formed during neutralization of ODFR, into  $\alpha$ -tocopherol<sup>18</sup>. Whereas, some of the proposed mechanisms for the cardioprotective effects of  $\text{Ca}^{+2}$  channel blockers are prevention of ischaemia-induced  $\text{Ca}^{+2}$  overload, reduction in the availability of  $\text{Ca}^{+2}$  to stimulate ATPase, proteases and lipases<sup>19</sup>, and prevention of lipid peroxidation<sup>13</sup>. We aimed to show if there is an additive cardioprotective effect with an antioxidant and a  $\text{Ca}^{+2}$  channel blocker.

Since ODFR are highly reactive with life spans shorter than microseconds, the only direct measurement method is electron spin resonance spectroscopy<sup>20</sup>. In practice, the generation of ODFR are detected by assays for biological molecules damaged by ODFR, usually lipids. As in most of the studies, we also accept MDA production as an index for ODFR activity.

It was reported that ascorbic acid might behave as an antioxidant or pro-oxidant depending on the ratio of  $\text{Fe}^{+3}$  to  $\text{Fe}^{+2}$  and ascorbic acid might prevent lipid peroxidation only at high concentrations<sup>21</sup>. In a previous study, ascorbic acid 250 mg.kg<sup>-1</sup>, i.v. effectively decreased the lipid peroxidation<sup>9</sup>. In our study, we obtained about 10 times increase in plasma ascorbic acid concentrations with a total dose of 100 mg.kg<sup>-1</sup>, i.v. and showed the prevention of lipid peroxidation in the supplemented groups. These results suggest that high dose ascorbic acid may effectively scavenge ODFR during and after CPB and offer a measure of protection to the myocardium.

The studies about cardioprotective effects of ascorbic acid and

diltiazem revealed conflicting results about cardiac enzyme levels. Although there are some data indicating decreased release of cardiac enzymes with ascorbic acid<sup>9</sup> and diltiazem<sup>22</sup> supplementation, the clinical importance of this finding could not be demonstrated. In the present study, cardiac enzyme levels were increased in all groups at the postoperative period. The lower levels in the group AA + D could not predict a better cardioprotection because the difference was not statistically significant. Although they are the traditional gold standards for myocardial infarction, the specificity of CPK, CK-MB and LDH are less than the specificity of CK-MB-M or troponin-T in differentiation of skeletal and cardiac muscle damage<sup>22,23</sup>. We therefore suggest that more specific markers should be used in such studies in order to detect even minor cardioprotection with the agents.

The source of the ODFR during myocardial ischaemia-reperfusion is still under debate. While Weisel and co-workers<sup>24</sup> observed that the levels of lipid peroxidation products were higher in the coronary sinus blood samples than in the arterial blood, the clinical study of Lazzarino and colleagues<sup>5</sup> revealed the opposite result. Also, in another clinical study, the postreperfusion MDA levels obtained from arterial and mixed venous blood were increased, despite no increase was present in coronary sinus blood<sup>20</sup>. In this study, the source of the MDA was thought to be the tissues other than myocardium, possibly lungs. The levels of MDA after declamping were both increased in arterial and coronary sinus blood samples in our study and no difference was found between arterial and coronary sinus samples at any time, which was consistent with several other studies<sup>25-27</sup>. These results suggest that the increase in the MDA levels is not because of its release only from the heart but also because of its release from the other organs affected by CPB.

Dingchao and co-workers<sup>9</sup> found CI significantly higher for the first 6 hours after the operation in the patients treated with high dose ascorbic acid. In another study, CI was increased significantly on the first postoperative day in patients given cardioplegia supplemented with diltiazem  $100 \mu\text{g}\cdot\text{kg}^{-1}$ <sup>28</sup>. Our study

revealed no significant difference between groups in terms of hemodynamic measurements. In all groups, CI was increased significantly after CPB and at the second postoperative hour when compared with the baseline measurements. So ascorbic acid and diltiazem offered no additional benefit in terms of CI measurements.

Reperfusion of the heart after an ischemic period may lead to potentially lethal arrhythmias<sup>29</sup>. It was suggested that ODFR and following lipid peroxidation might play a role in the genesis of ventricular arrhythmias<sup>30,31</sup>. There also appears studies in the literature supporting antiarrhythmic effects of diltiazem<sup>22,32</sup>. There is no article in the literature studying antiarrhythmic effects of ascorbic acid and diltiazem together. In the study of Hearse and Tosaki<sup>33</sup>, PBN (N-tert-butyl-alpha-phenylnitron), an antioxidant agent, and low perfusate  $\text{Ca}^{+2}$  concentration exerted additive antiarrhythmic effect over isolated perfused rat hearts and a concept of multiple interacting trigger mechanisms was suggested. In our study, we found a high incidence of VF after declamping in the group C which might be due to longer cross-clamping and CPB times when compared with the other studies. The number of VF was lower in the group AA and it decreased more with the addition of diltiazem to ascorbic acid. This result supported the additive antiarrhythmic effect between the antioxidants and decreased intracellular  $\text{Ca}^{+2}$  overload.

In our study, we observed the prevention of lipid peroxidation in ascorbic acid and ascorbic acid + diltiazem treated patients. The only positive result obtained by addition of diltiazem to high dose ascorbic acid was the decrease in the number of patients with VF after declamping. We concluded that despite the prevention of lipid peroxidation, the treatment regimens provided no measurable protection over myocardial ischaemia-reperfusion injury.

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