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THE PROTECTIVE EFFECTS OF CALCIUM ANTAGONIST AND FREE RADICAL SCAVENGER AGAINST MYOCARDIAL ISCHEMIC/REPERFUSION INJURY IN THE ISOLATED RAT HEART

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INDEXING WORDS

myocardial protection; calcium antagonist; free radical scavenger; isolated rat heart

SYNOPSIS

Using the isolated working rat heart model, efficacy of the calcium antagonist and free radical scavengers against the myocardial injury induced by ischemia and reperfusion was investigated. In the calcium antagonist series, diltiazem was added to St. Thomas' Hospital cardioplegic solution. After 35 minutes of ischemia (37C) and 30 minutes of reperfusion, the dose response curve of postischemic recovery of aortic flow showed the bell-shaped pattern and the addition of 0.4 mg/L of diltiazem significantly increased the final recovery of aortic flow from the control value of 45.3 ± 3.1% to 59.1 ±3.7% ($P < 0.01$). However, the higher dose of diltiazem reduced the postischemic recoveries of heart rate and aortic flow probably due to its side effects of negative inotropic and chronotropic effect. In the free radical scavengers series, 100 mg/L of superoxide dismutase (SOD) and

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10 mg/L of catalase (CAT) were added in the same experimental schedule. The addition of these enzymes significantly improved the postischemic recovery of aortic flow, and the best recovery was seen in the group receiving both SOD and CAT (72.9 ±3.9%, P <0.001). It was suggested that SOD and CAT have potential clinical application in preventing oxygen radical-mediated myocardial injury in the setting of open heart surgery.

INTRODUCTION

Preservation of myocardial fucntion during surgically induced global ischemia is a major concern of the cardiac surgeons performing cardiopulmonary bypass and interruption of coronary blood flow in cardiac operations. Although the current techniques of myocardial preservation using cold cardioplegia and topical cooling are generally satisfactory, their effect is limited in the group of patients who require prolonged interruption of coronary blood flow and in those patients with limited myocardial reserve.[14)]

To get a firmer understanding of the ischemic/reperfusion process and to improve the techniques of myocardial protection, various intensive investigations have been carried out in both clinical and experimental fields.[5,9,21)]

Recently, it has been suggested that termination of ischemia by resumption of coronary perfusion can result in a paradoxical extension of ischemic damage,[10)] and oxygen-derived free radicals and intracellular calcium overload can play a significant role in pathogenesis of the so-called reperfusion injury.[17)]

This study was designed to determine whether the addition of calcium antagonist or free radical scavengers to St Thomas' Hospital cardioplegic solution may prevent the myocardial injury induced by ischemia and reperfusion in the isolated, working rat heart.

MATERIALS AND METHODS

Isolated heart model

Male rats of the Sprague-Dawley strain weighing 300-400 g were anesthetized with an intraperitoneal injection of Nembutal

(20 mg/rat). Heparin sodium (5 mg/rat) was given intravenously via a tail vein. The heart was rapidly excised and dropped into Krebs-Henseleit solution at 4C. Contraction stopped within a few seconds.

The heart was then mounted on an insolated, working heart appratus as devised by Neely and associates[27] and modified by us (Fig. 1). Immediately after the aorta was cannulated and secured, nonrecirculating Langendorff retrograde perfusion was established at a pressure of 80 cmH_2O with Krebs-Henseleit bicarbonate buffer equilibrated with 95% O^2 and 5% CO_2 gas mixture. A pulmonary arteriotomy was performed and the left atrium was then cannulated via a pulmonary vein and secured. Perfusion fluid flew down from atrial reservoir located 18 cm above the heart and passed from the left atrium to the left ventricle providing a fixed filling

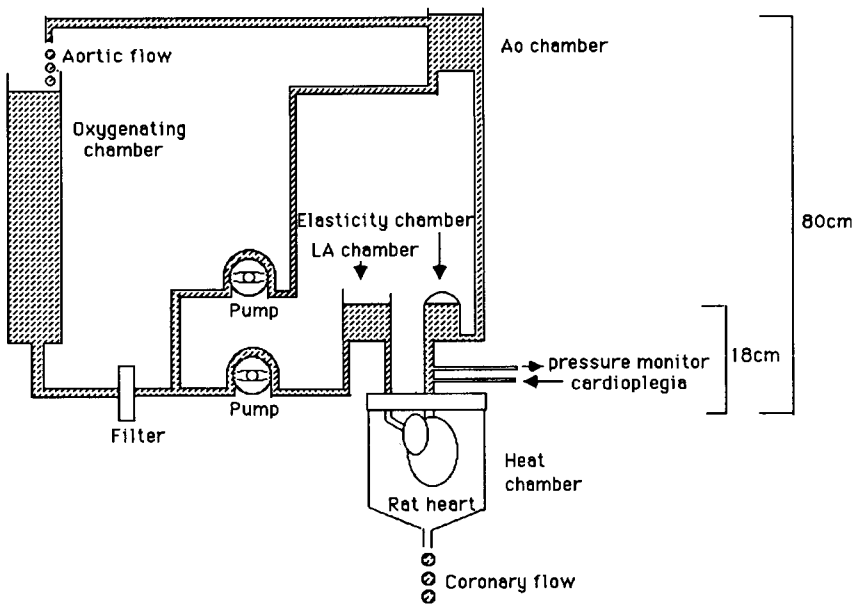

Fig. 1 Isolated perfused working heart model.
The rat heart is cannulated via the left atrium and the aorta and maintained in a thermostatically controlled chamber. The circulating solution is 37C Krebs-Henseleit bicarbonate buffer solution bubbled with 95% O_2 and 5% CO_2 gas mixture. 80 cmH_2O hydrostatic pressure of afterload and 18 cmH_2O hydrostatic pressure of preload are constantly established. All chambers are surrounded by a water jacket to maintain in 37C.

pressure, where it was ejected via the aortic cannula and an elasticity chamber against a 80 cmH_2O hydrostatic pressure. The mounted heart was placed in a water-jacketed chamber. The entire apparatus was water-jacketed and maintained at a temperature of 37C.

Aortic pressure was monitored and recorded with Gould-Statham physiologic pressure transducer connected to the aortic cannula and heart rate was also measured. Aortic flow was measured by timed collection of the perfusion fluid overflowed from aortic chamber and coronary flow was measured by collecting the coronary venous effluent which dropped from the isolated heart chamber at that time.

Experimental time course (Fig. 2)

All hearts subjected to ischemia underwent the same perfusion protocols. The major experimental variable was the additive to the cardioplegic solution.

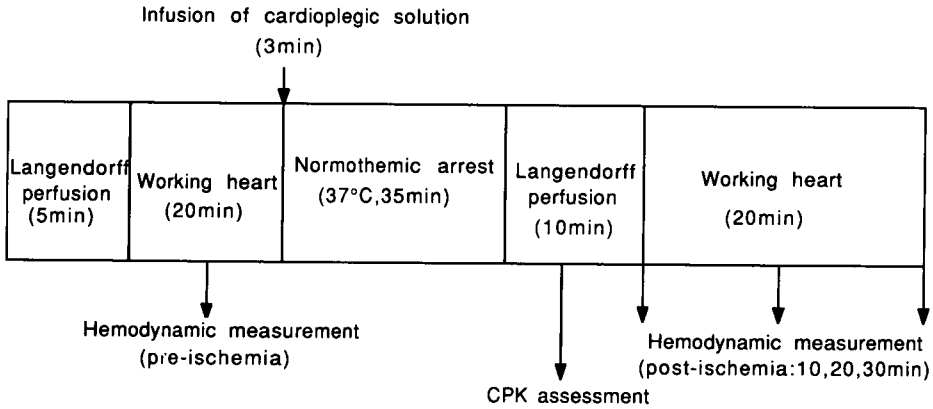

Fig. 2 Experimental time course.
Hearts were subjected to 5 minutes Langendorff perfusion and then 20 minutes initial control working perfusion. During this period, preischemic control hemodynamic values were obtained.
The hearts were then arrested by infusion of cardioplegic solution for 3 minutes and maintained in normothermic (37C) ischemic conditions for 35 minutes. Thereafter, Langendorff perfusion was resumed and coronary venous effluent was collected for creatinine kinase analysis during 10 minutes of reperfusion. The hearts were then subjected to working mode and postischemic hemodynamic values were measured at the point of 10, 20 and 30 minutes after reperfusion.

Hearts were sujected to aerobic Langendorff perfusion (5 minutes) and then an initial control working perfusion with oxygenated (95% O_2 and 5% CO_2) Krebs-Henseleit bicarbonate buffer (20 minutes, 37C). During this period, preischemic control hemodynamic values of heart rate, aortic pressure, aortic flow, and coronary flow were obtained. The atrial and aortic cannula were then clamped and hearts were arrested by coronary infusion of various cardioplegic solution via the aortic root. The cardioplegic solution was infused to the heart with the volume of 40 ml for 3 minutes. Infusion was then terminated and the ischemic hearts were maintained at 37C for 35 minutes.

At the end of ischemic period, the aortic clamp was removed and Langendorff perfusion was resumed. After 10 minutes empty beating interval, hearts were subjected to beat by releasing the atrial clamp and postischemic hemodynamic values were measured at the point of 10, 20, and 30 minutes after reperfusion.

Experimental groups

Seven groups of experimental hearts subjected to 35 minutes of normothermic ischemic with various cardioplegic protections were studied. Group 1 (control group) received 40 ml of the St. Thomas' Hospital cardioplegic solution composed of NaCl 110.0, KCl 16.0, $MgCl_2$ 16.0, $CaCl_2$ 1.2, $NaHCO_3$ 10.0 (in m mols/ liter), with pH 7.8 and osmolarity 285-300 mOsm/kg H_2O. In group 2, 3, and 4 (Ca-antagonist groups), diltiazem was added to the St. Thomas' Hospital cardioplegic solution at the concentration of 0.2, 0.4, and 0.6 mg/liter, respectively. The remaining groups were studied on the additive effect of free radical scavengers. Group 5 received the same amount of the St. Thomas' Hospital cardioplegic solution supplemented with 100 mg/liter of superoxide dismutase (SOD) and group 6 supplemented with 10 mg/liter of catalase (CAT) and group 7 with both SOD and CAT. SOD was obtained from Ube Industries, Japan and its specific activity was 3,300 units/mg protein. CAT was obtained from Boehringer Mannheim, Ltd., West Germany and its specific activity was 65,000 units/mg protein.

Expression of results

In functional studies, the postischemic recovery of aortic flow, coronary flow, peak aortic pressure, and heart rate were

expressed as a percent of the preischemic control value in each heart.

During the postischemic Langendorff period, coronary venous effulent aliquots were collected for analysis of enzyme and total leakage of creatine kinase was measured by the method of Oliver.[28]

At the end of the experiment, the hearts were removed from the apparatus, and their weights were measured as wet weight. The hearts were then dried to constant weight to be measured as dry weight, and tissue water content was calculated according to the following formula:

$$\text{tissue water content}(\%) = \frac{\text{wet weight}(g) - \text{dry weight}(g)}{\text{wet weight}(g)} \times 100$$

All data analyzed by Student's t test. The results were expressed as mean ± standard error of the mean. A p-value of 0.05 or less was considered statistically significant.

RESULTS

Functional assessment

The additional effects of diltiazem and free radical scavengers in functional recovery of ischemic heart were shown in Fig. 3,4,5 and Table 1.

Compared with the control group, the postishcemic recovery of heart rate in diltiazem-treated groups was lower after 10 minutes of reperfusion and then gradually increased. In group 2 and 3, it finally reached at the level of group 1 after 30 minutes of reperfusion, but group 4 resulted in significantly poor recovery of heart rate (Fig. 3).

In the postischemic recovery of aortic flow afforded by diltiazem, the dose-response curve showed the bell-shaped pattern and the addition of 0.4 mg/liter of diltiazem (group 3) increased the final recovery of aortic flow from the control value of 45.3 ±3.1% to 59.1 ±3.7% ($P < 0.01$). The result of postischemic recovery of coronary flow was also parallel to the recovery of aortic flow (Fig. 4).

Fig. 5 shows the results of postischemic functional recovery after 30 minutes of reperfusion afforded by free radical scavengers. The pattern of delayed recovery of heart rate which was seen

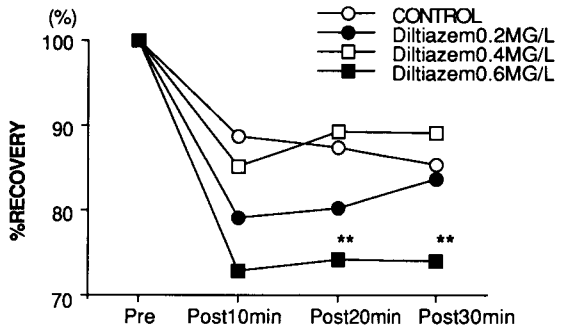

Fig. 3 Postischemic recovery of heart rate in the groups of diltiazem cardilplegia. The recovery is expressed as a percent of the preischemic control value.
Open circles, group 1 receiving St. Thomas' Hospital cardioplegic solution (STHCS). Closed circles, group 2 receiving STHCS with 0.2 mg/L of diltiazem. Open squares, group 3 receiving STHCS with 0.4 mg/L of diltiazem. Closed squares, group 4 receiving STHCS with 0.6 mg/L of diltiazem.(**P <0.005)

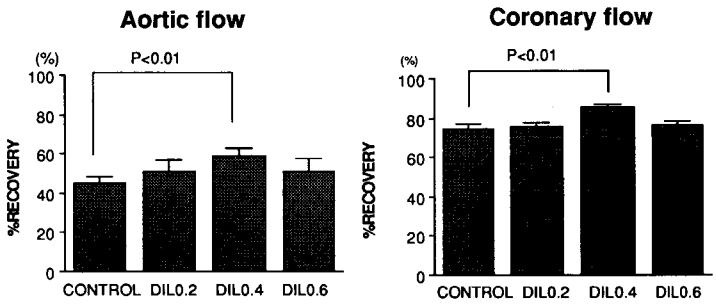

Fig. 4 Postischemic recovery of aortic and coronary flow in the groups of diltiazem cardioplegia after 30 minutes of reperfusion. The recovery is expressed as a percent of the preischemic control value.
CONTROL, group 1 receiving St. Thomas' Hospital cardioplegic solution (STHCS). DIL0.2, group 2 receiving STHCS with 0.2 mg/L of diltiazem. DIL0.4, group 3 receiving STHCS with 0.4 mg/L of diltiazem. DIL0.6, group 4 receiving STHCS with 0.6 mg/L of diltiazem.

in diltiazem-treated groups was not found in free radical scavenger groups and there were no significant differences in the recovery of heart rate between control and any free radical scavenger groups.

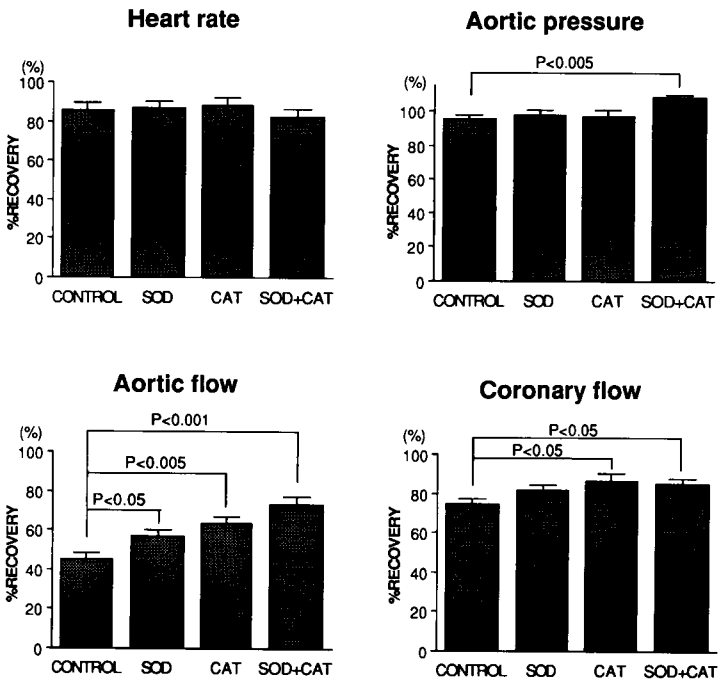

Fig. 5 Postischemic functional recovery in the groups of free radical scavenger cardioplegia after 30 minutes of reperfusion. The recovery is expressed as a percent of the preischemic control value.
CONTROL, group 1 receiving St. Thamas' Hospital cardioplegic solution (STHCS). SOD, group 5 receiving STHCS with 100 mg/L of SOD. CAT, group 6 receiving STHCS with 10 mg/L of CAT. SOD+CAT, group 7 receiving STHCS with both SOD and CAT.

In the postischemic recovery of aortic flow, the addition of superoxide dismutase and/or catalase to St. Thomas' Hospital cardioplegic solution (group 5, 6 and 7) significantly improved the functional myocardial protection. The best recovery was obtained by the combined administration of these enzymes (group 7) which showed marked improvement from the control value of 45.3 ±3.1% to 72.9 ± 3.9% (P <0.001). Similar recovery patterns were found in coronary flow data and group 6 and 7 showed significantly higher recovery.

Finally, the postischemic recovery of cardiac output and stroke volume in the hearts treated with both superoxide dismutase and catalase (71.3 ±3.7%, 85.8 ±4.2%, group 7) were better than in any other group and the difference was significant (p <0.005) (Table 1).

Table 1 The Postischemic recovery of various parameters of cardiac function after 35 minutes ischemia and 30 minutes reperfusion.

Group		Heart rate (% control)	Aortic pressure (% control)	Aortic flow (% control)	Coronary flow (% control)	Cardiac output (% control)	Stroke vloume (% control)
1)St.Thomas solution	n=9	85.3±4.5	95.1±2.3	45.0±3.1	74.3±2.9	50.9±2.9	61.3±3.3
2)Diltiazem0.2mg/L	n=7	83.5±5.5	98.3±4.3	51.0±5.7	75.8±1.9	56.3±4.6	69.2±6.5
3)Diltiazem0.4mg/L	n=7	89.0±1.5	93.8±1.7	59.1±3.7 **	85.3±1.9 **	62.3±3.3 *	69.5±4.5
4)Diltiazem0.6mg/L	n=7	74.0±3.7 §	104.4±2.3 *	51.1±6.3	76.5±1.9	56.3±5.2	75.1±3.5 *
5)SOD 100mg/L	n=9	87.0±3.4	98.0±2.5	56.9±3.5 *	81.8±2.6	60.2±2.9 *	70.3±4.0
6)CAT 10mg/L	n=7	88.4±3.5	97.0±4.0	63.1±3.5 §	86.0±3.9 *	69.4±3.3 §	79.1±4.8 **
7)SOD+CAT	n=9	82.4±3.9	107.9±1.5 §	72.9±3.9 §§	84.9±2.8 *	71.3±3.7 §§	85.8±4.2 §§

SOD: superoxide dismutase. CAT: catalase. Values are mean ± SE.
*p<0.05, **p<0.01, §P<0.005, and §§p<0.001 versus group1.

Enzymatic assessment (Fig. 6)

In the hearts treated with diltiazem (group 2, 3 and 4), there were moderate decreases in the total leakage of creatine kinase during 10 minutes of reperfusion, which did not differ from the control group (group 1). In free radical scavenger groups, group 6 and 7 (340.8 ±83.0, 430.7 ±80.1 mU/10min/heart) strongly reduced the postischemic creatine kinase leakage and they were significantly less than that of group 1 (1318.5 ±329.2 mU/10min-/heart).

Tissue water content

Tissue water content after ischemia was 82.3 ±0.3% in the control group. Any hearts treated with diltiazem or free radical

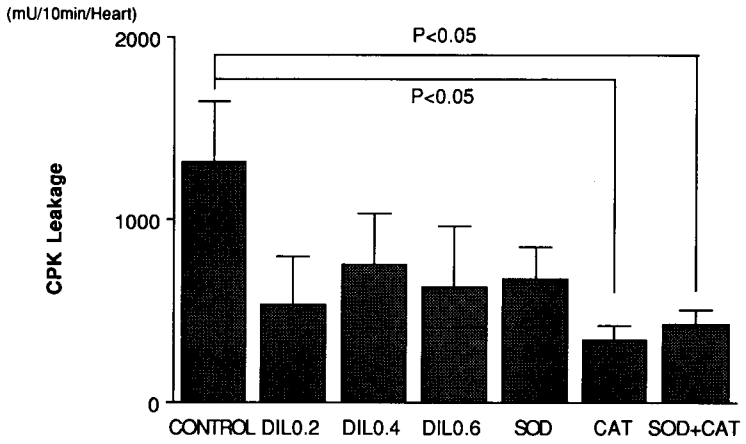

Fig. 6 Total leakage of creatine kinase during 10 minutes of reperfusion after 35 minutes of normothermic ischemia.

scavengers showed less values than that of group 1 as follows: group 2, 80.8 ±0.3%; group 3, 80.9 ± 0.3%; group 4, 79.8 ±0.3%; group 5, 79.7 ±0.3%; group 6, 78.9 ± 0.4%; group 7, 80.6 ±0.3%. These differences were statistically significant ($P < 0.001$).

DISCUSSION

The present study confirmed that the calcium antagonist or free radical scavengers added to St. Thomas' Hospital cardioplegic solution was able to enhance the myocardial protection against normothermic ischemia in the isolated, working rat heart. The calcium antagonist study suggested that the protective effect of diltiazem on myocardium was not linear with concentration and the higher dose of diltiazem did not always improve the postischemic functional recovery. Further, the free radical scavenger study confirmed both functionally and enzymatically that the combined addition of superoxide dismutase (SOD) and catalase (CAT) showed the best protective effect in all of these experimental groups.

Protective effect of diltiazem

Since intracellular calcium accumulation was implicated as an

important mediator of myocardial injury during ischemia and reperfusion,[13,16,38] calcium antagonist has been proposed by a number of investigators for protection of the ischemic myocardium.[2,3,22,23,30,33] It is mainly based on the idea that calcium entry blockers inhibit the influx of calcium into the myocardial cell, reduce the mitochondrial calcium overload, and maintain the inonic homeostasis to preserve the high-energy phosphate production and support the postischemic myocardial function.[14] Moreover, calcium antagonist has another beneficial effect on myocardial oxygen supply by coronary vasodilation and myocardial oxygen demand by systemic vasodilation and decreased left ventricular afterload.[8] The present study has shown the efficacy of diltiazem against the myocardial ischemia, and the additive dose of 0.4 mg/liter of diltiazem in St. Thomas' Hospital cardioplegic solution significantly increased the postischemic functional recovery. However, the dose of 0.6 mg/liter did not differ from the effect of myocardial protection in the control group, and the dose-response curve showed the bell-shaped pattern. This characteristic dose-response curve was also pointed out by Yamamoto and Hearse[39] and the reason of the bell-shaped pattern is thought to be probably due to the negative inotropic and chronotropic effect of diltiazem on cardiac muscle.[15]

Some recent investigators have also demonstrated the advanced efficacy of calcium antagonist in myocardial protection. But their opinions are widely different at the points of the choice of calcium antagonists, the best effective temperature, the optimal timing of administration, the opitimal dosage and others.[7,15,20,26,36,39] This study disclosed that the higher dose of diltiazem would not be effective in myocardial protection, because of its side effects, namely, negative inotropic and chronotropic effects.

Protective effect of free radical scavenger

Recent studies on the relation of oxygen metabolities and ischemic damage have contributed to further recognition of the generation or reactivity of cytotoxic oxygen free radicals.[11,14,32]

Following the onset of myocardial ischemia, there is a rapid decrease in myocaridal pH, and with the decrease in molecular oxygen, there is an increase in reducing equivalents (NADH and

$FADH_2$), which favors the univalent reduction of oxygen. These changes in myocardium result in an increased production of oxygen free radicals, namely, superoxide anion($\cdot O_2$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\cdot OH$) (Fig. 7). These species of oxygen free redicals are highly reactive and known to be cytotoxic. In addition, during the reperfusion period, molecular oxygen is reintroduced to the ischemic myocardium, and a bursting production of oxygen free radicals occurs to lead the extensive myocardial injury and dysfunction.[6,24,35,37]

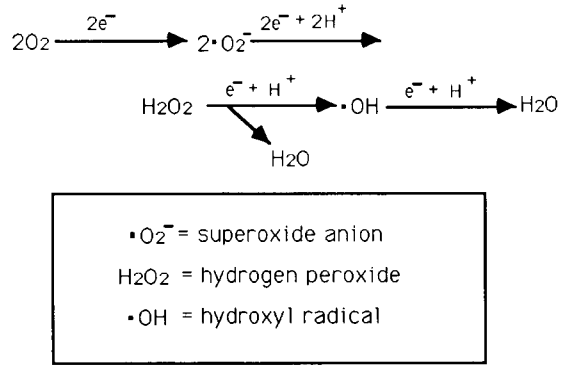

Fig. 7 The oxygen free radical system.

The intracellular biologic defenses against oxygen free radical-mediated tissue damage include SOD, an enzyme that catalyzes the coversion of superoxide anion to hydrogen peroxide and molecular oxygen, and two additional cytoplasmic enzymes, CAT and glutathione peroxide that convert hydrogen proxide to water and molecular oxygen. However, there are no host enzyme systems to scavenge hydroxyl radical. Under normal physiologic condition, hydroxyl radicals would not be formed, because their production requires both superoxide anion and hydrogen peroxide, which are normally scavenged as above. But, in pathologic conditions, once hydroxyl radicals are excessively generated, these destructive radicals can attack the unsaturated fatty acid chain of cell membrane to produce lipid peroxidation, which result in an increase in membrane fluidity, increasing permeability, and loss of membrane integrity.[35,37]

In this study, we indirectly demonstrated that oxygen free radicals were the mediators of ischemia and reperfusion injury and that the administration of the exogeneous SOD and CAT in

cardioplegic solution could reduce the ultimate extent of myocardial injury in the isolated heart. This main protective mechanism is probably due to prevention of hydroxyl radicals formation.

The efficacy of SOD and CAT against ischemic/reperfusion injury is also studied in experimental regional and global ischemic heart models by some other investigators.[12,18,19,25,29] Shlafer and associates[31] have reported that these enzymes improved the left ventricular fucntion after 2 hours of ischemia at 27C in isolated rabbit heart model and suggested the possibility of their protective efficacy at the lower myocardial temperatures that are encountered more rountinely in clinical practice.

Thus, SOD and CAT have potential clinical application in preventing oxygen radical-mediated myocardial injury in the setting of open heart surgery, but more definitive proof of the efficacy under more relevant clinical conditions including their safety should be submitted in the near future.

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