In Vitro Study on Contribution of Oxidative Metabolism of Isolated Rabbit Heart Mitochondria to Myocardial Reperfusion Injury

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SUMMARY. The present study was performed to clarify oxygen-induced damage following myocardial reperfusion, using three mitochondrial preparations from isolated rabbit hearts (non-ischemic hearts and those subjected to 40 and 90 minutes of normothermic global ischemia). The viability of mitochondria was evaluated by adenosine triphosphate generation. The extent of mitochondrial injury produced by reactive oxygen metabolism was assessed by the intensity of hydroxyl radical signal detected with electron spin resonance spectroscopy and the reduction of coenzyme Q10 level. The greatest oxygen-induced injury was observed in 40-minute ischemic mitochondria exposed to pure oxygen. The use of superoxide dismutase and catalase satisfactorily prevented the oxygen-induced injury. Moreover, the net adenosine triphosphate generation of the 40-minute ischemic mitochondria was comparable to that of the nonischemic mitochondria without the enzymes. These results suggest that reperfusion of the ischemic myocardium with viable mitochondria is deleterious, because mitochondria are susceptible to injury resulting from oxidative metabolism, and that the use of superoxide dismutase, together with catalase, is beneficial for the restoration of cardiac function after ischemia. (Circ Res 55: 168–175, 1984)

REVASCULARIZATION, both surgical and nonsurgical, after coronary occlusion, is an established therapeutic procedure for patients with acute myocardial infarction. However, the contribution of successful revascularization to myocardial salvage is still controversial. There is no doubt that irreversible myocardial damage will develop unless reperfusion is performed within several hours. Although some authors suggest that reperfusion should be carried out even after extended periods of coronary occlusion (Maroko et al., 1972; Ginks et al., 1972; Puri, 1975; Reimer et al., 1977), others have drawn attention to the possible detrimental effects of reperfusion after coronary occlusion (Lang et al., 1974; Bresnahan et al., 1974; Banka et al., 1974; Bulkley and Hutchins, 1977; Smith et al., 1978).

Despite the evidence of functional, metabolic, and structural abnormalities during reperfusion, the precise mechanisms of reperfusion injury remain obscure because reperfusion exhibits multifactorial effects on the ischemic myocardium. Thus, it is necessary to examine the characteristic events occurring in the myocardium and evaluate the factors involved in the recovery of myocardial function during the post-perfusion period.

Reperfusion results in abrupt delivery of oxygen and substrates to the myocardium, which the mitochondria utilize principally for energy production; thus, the initiation of mitochondrial respiration is considered a characteristic phenomenon of reperfusion. On the other hand, mitochondria are regarded as being the most susceptible to ischemia among the cellular components, and it appears that reperfusion itself has deleterious effects on mitochondrial function (Kane et al., 1975; Sugiyama et al., 1980b). Since the recovery of myocardial contraction after ischemia is ultimately dependent on the available energy supply, the inability of the mitochondria to produce sufficient energy would soon result in myocardial dysfunction.

Therefore, we investigated the relationship between mitochondrial oxidative metabolism and cardiac reperfusion injury, using in vitro models. The efficacy of activated oxygen scavengers, superoxide dismutase (SOD), and catalase, in protecting against such injury was also examined.

Methods

Rabbits of both sexes, weighing 2–3 kg, were lightly anesthetized with sodium pentobarbital injected intravenously, and their hearts were rapidly excised. Three normothermic mitochondrial preparations were studied: nonischemic, and those obtained from hearts subjected to 40 and 90 minutes of global ischemia.

For the isolation of rabbit heart mitochondria (Chance and Haghia, 1963), the ventricle was minced in ice-
cooled 0.25 M sucrose, and suspended in isolation medium containing 0.25 M sucrose, 5 mM Tris-HCl buffer, and 0.25 mM ethylenediaminetetraacetic acid (EDTA) (pH 8.0). Next, 50 mg of bacterial alkaline protease (Nagase Industrial Company) were added, and the mixture was stirred slowly for 20 minutes. The material then was gently homogenized and the suspension was centrifuged at 500 g for 5 minutes. The supernatant layer was centrifuged at 6500 g for 10 minutes. The dark brown sediment was suspended in a small amount of isolation medium (pH 7.4) at 2–4°C.

**Incubation of Mitochondria**

State 4 respiration was initiated by incubation of 1.0 ml of the mitochondrial suspension in a reaction medium (0.25 M sucrose, 5 mM Tris, 20 mM inorganic phosphate, 0.25 mM MgCl₂, 0.25 mM EDTA, and 5 mM succinate as a substrate at pH 7.4 with 6 N KOH) which had been equilibrated with oxygen, room air, and nitrogen in test tubes at 30°C. Transition from state 4 to state 3 respiration was induced by the addition of 0.1 ml of 0.5 M adenosine diphosphate (ADP). The same amounts of ADP and 0.1 ml of 1.0 M succinate were added 15 minutes after the start of state 3 respiration, since these concentrations were considered large enough to allow the mitochondria to generate the optimum amount of ATP. In state 1 respiration, the mitochondria were incubated with isolation medium at pH 7.4.

The volume of the subsequent mitochondrial suspensions was 10.0 ml at a protein concentration of 0.45–0.96 mg/ml, determined by the method of Lowry et al. (1951). Mitochondria were continuously aerated with each gas during the 30-minute incubation. Aliquots (1.0 ml) of the mitochondrial suspensions were removed just before and at 10-minute intervals after the start of incubation in order to determine the mitochondrial coenzyme Q₁₀ content and ATP generation.

**Identification of Hydroxyl Radical**

Since many investigators have failed to detect short-lived oxygen radicals directly, by electron spin resonance (ESR) spectroscopy, the ESR-spin trapping technique, using 5,5′-dimethyl-1-pyrroline-1-oxide (DMPO, Sigma) as a spin-trapping agent, was employed to detect oxy-radicals being generated in respiring mitochondria.

In this experiment, 0.5-ml aliquots of mitochondria (8 mg of protein/ml) were incubated in 4.5 ml of the above-mentioned reaction medium to which was added 0.1 M DMPO purified by treatment with activated neutral charcoal. The mitochondria suspension was incubated for 5 minutes to obtain the optimal ESR signal. ESR spectra were recorded with a JEOL JES-1X spectrometer at room temperature.

**Determination of Coenzyme Q₁₀**

For determination of the mitochondrial coenzyme Q₁₀ content (Ikenoya et al., 1981), 0.5 ml of mitochondrial suspension was placed into a test tube containing 8 ml of ethanol/n-hexane (3:5 by volume). The tube then was shaken to extract the coenzyme Q₁₀, which was dried under a stream of nitrogen and analyzed by high performance liquid chromatography.

**Determination of ATP Generation**

State 3 respiration was terminated by placing the mitochondrial suspension (0.5 ml) in a test tube containing 2.5 ml of 0.6 N perchloric acid (PCA). After centrifugation of the PCA extract, the supernatant was adjusted to pH 7.0 and was used for determining ATP according to the enzymatic procedure (Bücher, 1947) using a test combination (Boehringer Manheim Company).

**SOD and Catalase**

Shortly before the incubation, 1 μM bovine liver SOD (specific activity of 3000 U/mg protein) and 0.2 μM bovine liver catalase (specific activity of 2000 U/mg protein) were added to the reaction medium. Both agents were obtained from Sigma Chemical Co.

**Statistical Analysis**

Student's t-test for paired data was used for statistical analysis, and results were expressed as mean ± sd.
mitochondria were calculated in relation to the mean signal height of the 40-minute ischemic group.

Relative intensity of ESR signal in the 40-minute ischemic group was significantly greater than those of nonischemic and 90-minute ischemic groups (Table 1). The hydroxyl radical signal during state 3 respiration of the 40-minute ischemic mitochondria was not as high as the additional signal, which is independent of the hydroxyl radical and likely to be associated with reactive oxygen intermediates, but could not be identified in these experiments (Fig. 2A).

**TABLE 1**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Relative intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonischemia (n = 8)</td>
<td>0.77 ± 0.08</td>
</tr>
<tr>
<td>40-Min ischemia (n = 8)</td>
<td>1.00 ± 0.17*†</td>
</tr>
<tr>
<td>90-Min ischemia (n = 8)</td>
<td>0.56 ± 0.10</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD.
* Significant difference from nonischemic group; P < 0.01.
† Significant difference from 90-minute ischemic group; P < 0.001.

During state 4 respiration, room air had no significant effect on the hydroxyl radical signal of the 40-minute ischemic mitochondria (Fig. 2B), whereas exposure to nitrogen completely inhibited the ESR signal (Fig. 2C). No DMPO adducts were formed during state 1 respiration, even when 40-minute ischemic mitochondria were continuously exposed to oxygen (Fig. 2D).

The incubation of 40-minute ischemic mitochondria treated with SOD and catalase under oxygen exposure markedly suppressed the appearance of hydroxyl radical signal, during state 4 (Fig. 3A) and state 3 respiration (Fig. 3B).

It is known that hydroxyl radical is generated dramatically in the H2O2 generation system in the presence of a catalytic amount of iron. Therefore, in order to examine whether the buffers used in this study contained a sufficient amount of iron to influence the intensity of DMPO-OH adduct signal, we conducted ESR measurements with the buffer containing an excess amount of H2O2 (50 mM). Since DMPO-OH adduct signal was not detected, we may assume that the buffer used in our experiment did not contain a sufficient amount of iron to change the ESR signal significantly.

In addition, the intensity of the DMPO-OH adduct signal in ESR measurement of the buffer treated with Chelex 100 resin was nearly identical to that of the buffer untreated with the same resin.

**Level of Coenzyme Q10 Content in Mitochondria**

As shown in Table 2, the coenzyme Q10 content in mitochondria during state 3 respiration decreased in every gas phase among the three groups. Aeration with oxygen of nonischemic mitochondria resulted in a significant reduction in the coenzyme Q10 content, not only compared with the control value, but also with aeration using room air.

Coenzyme Q10 content of the 40-minute ischemic group, whose control value was 77% that of the nonischemic group, was reduced by 37% of the control value after 30-minute incubation in oxygen. A significant difference was observed between the oxygen and room air gas phases as early as 10 minutes after incubation. Levels of coenzyme Q10

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**Figure 2.** ESR spectra of the DMPO adducts observed in 40-minute ischemic mitochondria generated during state 3 respiration with oxygen (panel A), during state 4 respiration with either room air (panel B), or nitrogen (panel C), or during state 1 respiration with oxygen (panel D). Each reaction mixture contains 0.8 mg of mitochondrial protein/ml. ESR conditions are the same as shown in Figure 1.

**Figure 3.** ESR spectra of the DMPO adducts obtained from 40-minute ischemic mitochondria treated with SOD and catalase under oxygen exposure in state 4 respiration (panel A) and state 3 respiration (panel B). Arrows indicate the appearance of the hydroxyl radical signal. The mitochondria used here are the same as those in Figure 1A. Reaction mixtures contain 1 μM SOD and 0.2 μM catalase. ESR conditions are the same as shown in Figure 1.
content during aeration with nitrogen showed mid-
values between aeration with room air and oxygen.

The reduction of coenzyme Q_{10} content in the 90-
minute ischemic group, whose control value was
44% that of the nonischemic group, was inhibited
considerably in every gas phase compared with the
other two groups. Whereas aerations with oxygen
and nitrogen showed significant reductions in coen-
zyme Q_{10} content from the control values 30 min-
utes after incubation, aeration with room air did not.
In this group, the differences in coenzyme Q_{10} content
among the three gas phases were not significant.

In the 40-minute ischemic mitochondria, changes
in the level of coenzyme Q_{10} during state 4 respira-
tion were similar to those during state 3. In contrast,
no changes in any of the gas phases were seen
during state 1 respiration (Table 3).

Table 4 shows the effect of SOD and catalase on
the change in coenzyme Q_{10} content of mitochondria
during state 3 respiration. A significant reduction in
coenzyme Q_{10} content was observed after 30 min-
utes of incubation with oxygen, but not with room
air. Thus, the administration of the enzymes to 40-
minute ischemic mitochondria, which exhibited the
largest hydroxyl radical generator, satisfactorily pre-
served coenzyme Q_{10} under aerobic conditions, but
had no effect on anaerobically respiring mitochon-
dria.

Mitochondrial ATP Generation

The time courses of mitochondrial ATP generation
during state 3 respiration are plotted in Figure 4. A
significant difference in ATP generation between
room air and oxygen exposure, was seen 20–30

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### Table 2

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>10 min</th>
<th>20 min</th>
<th>30 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonischemia (n = 6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxygen exposure</td>
<td>3.80 ± 0.35</td>
<td>2.97 ± 0.27 ‡</td>
<td>2.60 ± 0.21 †</td>
<td>2.45 ± 0.27 ‡</td>
</tr>
<tr>
<td>Room air exposure</td>
<td>3.39 ± 0.30</td>
<td>3.07 ± 0.25 ‡</td>
<td>2.93 ± 0.25 †</td>
<td>2.79 ± 0.24 ‡</td>
</tr>
<tr>
<td>Nitrogen exposure</td>
<td>3.23 ± 0.31 †</td>
<td>2.97 ± 0.32 †</td>
<td>2.59 ± 0.24 ‡</td>
<td></td>
</tr>
<tr>
<td>40-Min ischemia (n = 6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxygen exposure</td>
<td>2.91 ± 0.27</td>
<td>2.25 ± 0.22 ‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Room air exposure</td>
<td>3.66 ± 0.19</td>
<td>2.49 ± 0.19 *</td>
<td>2.39 ± 0.23 †</td>
<td>2.03 ± 0.22 ‡</td>
</tr>
<tr>
<td>Nitrogen exposure</td>
<td>2.34 ± 0.26 †</td>
<td>2.14 ± 0.22 ‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>90-Min ischemia (n = 6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxygen exposure</td>
<td>1.69 ± 0.21</td>
<td>1.46 ± 0.14</td>
<td>1.37 ± 0.17 *</td>
<td>1.29 ± 0.19 †</td>
</tr>
<tr>
<td>Room air exposure</td>
<td>1.64 ± 0.21</td>
<td>1.49 ± 0.24</td>
<td>1.42 ± 0.20</td>
<td>1.39 ± 0.14 *</td>
</tr>
<tr>
<td>Nitrogen exposure</td>
<td>1.63 ± 0.20</td>
<td>1.54 ± 0.17</td>
<td>1.42 ± 0.20</td>
<td>1.39 ± 0.14 *</td>
</tr>
</tbody>
</table>

Values (nmol/mg protein) are expressed as mean ± SD.

* † ‡ Significant difference from control; P < 0.02, P < 0.01, P < 0.001, respectively.

§ † ‡ Significant difference from room air at the same incubation period; P < 0.02, P < 0.01, P < 0.001, respectively.

### Table 3

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>10 min</th>
<th>20 min</th>
<th>30 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>State 4 respiration (n = 6)</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Oxygen exposure</td>
<td>3.02 ± 0.35</td>
<td>2.42 ± 0.16 ‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Room air exposure</td>
<td>2.89 ± 0.31</td>
<td>2.78 ± 0.27</td>
<td>2.56 ± 0.18 *</td>
<td></td>
</tr>
<tr>
<td>Nitrogen exposure</td>
<td>2.75 ± 0.24</td>
<td>2.55 ± 0.28</td>
<td>2.33 ± 0.27 †</td>
<td></td>
</tr>
<tr>
<td>State 1 respiration (n = 6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxygen exposure</td>
<td>2.87 ± 0.16</td>
<td>2.76 ± 0.26</td>
<td>2.74 ± 0.31</td>
<td>2.75 ± 0.21</td>
</tr>
<tr>
<td>Room air exposure</td>
<td>2.76 ± 0.31</td>
<td>2.79 ± 0.33</td>
<td>2.78 ± 0.27</td>
<td></td>
</tr>
<tr>
<td>Nitrogen exposure</td>
<td>2.85 ± 0.28</td>
<td>2.84 ± 0.20</td>
<td>2.84 ± 0.23</td>
<td></td>
</tr>
</tbody>
</table>

Values (nmol/mg protein) are expressed as mean ± SD.

* † ‡ Significant difference from control; P < 0.02, P < 0.01, P < 0.001, respectively.

§ † ‡ Significant difference from room air at the same incubation period; P < 0.01, P < 0.001, respectively.
minutes after incubation in the nonischemic mitochondria. ATP generation during nitrogen exposure was less than one-third of that observed during room air exposure, probably due to a slight oxygen contamination.

Similar time courses were observed in the 40-minute ischemic group. However, the difference in ATP generation between these two aerobic conditions was more obvious 20 and 30 minutes after incubation than in the nonischemic group. Little ATP generation was seen in the 90-minute ischemic group under aerobic condition.

The administration of SOD and catalase, enhanced ATP generation in 40-minute ischemic mitochondria under aerobic conditions (Fig. 5). The impairment of oxidative phosphorylation seen with oxygen was not observed in this group when the enzymes were added. Moreover, the net ATP generation under aerobic conditions with the enzymes was comparable to that in the nonischemic group aerated with room air. However, the enzymes had no effect on ATP generation of mitochondria aerated with nitrogen.

Discussion

The assumption that myocardial reperfusion causes unfavorable results has increasingly pervaded the field of cardiac research. Despite the accumulation of evidence concerning reperfusion injury, no exact mechanisms have yet been confirmed. The harmful effect of reoxygenation on the myocardium has been discussed from the aspect of contractile abnormality (Bing et al., 1971, 1976), ultrastructural damage (Feuvray and Leiris, 1975; Hearse et al., 1975, 1978; Ganote and Kaltenbach, 1979), and enzyme release (Ganote et al., 1976, Hearse et al., 1978; Ganote and Kaltenbach, 1979).

Our interest in reperfusion injury has been focused on the deterioration of the mitochondria. In our study, mitochondria whose respiration had been terminated as a result of oxygen and substrate deprivation during ischemia and/or preparation of mitochondria at low temperature showed an apparent impairment of phosphorylative activity in the presence of high concentrations of oxygen. Therefore, oxygen itself appears to be a major determinant of reperfusion injury for the following reasons.
zyme Q10, because of its potent antioxidant activity, its antioxidative action (Takayanagi et al., 1980; Gestetner et al., 1981; recent studies have stressed its importance in the prevention of peroxidation.

The amount of antioxidants terminates the propagation of the oxidative chain reaction, which causes irreversible cell death unless an adequate supply of antioxidants is available. These antioxidants may be found in cellular organelles (Hunter et al., 1963; Bovet et al., 1973), which are highly reactive free radicals (Feeney and Berman, 1974; Boveris and Chance, 1973). Activated oxygen consists of superoxide anion (\( \cdot O_2^- \)), hydrogen peroxide (\( H_2O_2 \)), hydroxyl radical (\( \cdot OH \)), and singlet oxygen (\( \cdot O_2 \)) (Maestro, 1980; Vladimirov et al., 1980).

Any activated oxygen can initiate peroxidation with such cellular components as phospholipids, protein constituents, and nucleotides. Above all, phospholipids containing abundant unsaturated free fatty acids are easily peroxidized. Since phospholipids are the principal element of cytoplasmic structures and biomembranes, their peroxidative cleavage leads to the loss of homeostasis and specific activity of cellular organelas (Hunter et al., 1963; Sledge et al., 1965; Bidlack and Tappel, 1973), which causes irreversible cell death unless an adequate amount of antioxidants terminates the propagation of peroxidation.

An earlier report (Mellors and Tappel, 1966) suggested that reduced coenzyme Q\(_{10} \) function as an antioxidant, and recent studies have stressed its antioxidative action (Takayanagi et al., 1980; Sugiyama et al., 1980a). Thus, the endogenous coenzyme Q\(_{10} \), because of its potent antioxidant activity, is reduced by respiratory-dependent lipid peroxidation.

The beneficial effect of coenzyme Q\(_{10} \) on mitochondrial function involves more than the antioxidative action; it might be related more closely to structural effects (Kobayashi et al., 1980) and activation of the respiratory enzymes of the electron transfer system (Szarkowska, 1966; Norling et al., 1974). Therefore, the depletion of coenzyme Q\(_{10} \) triggered by lipid peroxidation might affect mitochondrial respiratory function and the integrity of the membrane structure, and eventually impair phosphorylation activity; it is possible that activated oxygen directly inhibits mitochondrial respiratory function. Therefore, the parallel observed in the present study between the reduction of coenzyme Q\(_{10} \) along with the formation of hydroxyl radical and the reduction in ATP generation, seems to be of importance.

At present, a persuasive discussion of the mechanism of the coenzyme Q\(_{10} \) reduction in the mitochondria under nitrogen atmosphere, in which no significant generation of oxygen radicals was observed, is impossible. However, the present study, as well as other recent works demonstrating a considerable decrease in coenzyme Q\(_{10} \) in myocardium and/or mitochondria due to regional and global myocardial ischemia (Yamasawa et al., 1980; Chiba, 1982), seems to support the following speculation: State 3 and state 4 respiration of the mitochondria in a nitrogen atmosphere in the presence of available substrates is identical to the environment of ischemic myocardium. Thus, an excessive oxidation-reduction state of the respiratory chain of mitochondria may have caused the coenzyme Q\(_{10} \) reduction by an unknown mechanism.

High oxygen tension was demonstrated to increase \( H_2O_2 \) release of mitochondria (Bovers and Chance, 1973; Turrens et al., 1982). Our study also suggested that oxygen-induced mitochondrial injury was caused by the enhancement of mitochondrial respiration during excessive oxygen exposure. In addition, the fact that formation of hydroxyl radical and reduction of coenzyme Q\(_{10} \) was not observed in state 1 respiration, in which oxygen consumption is negligible due to the absence of exogenous substrate despite the presence of available oxygen, indicates that oxygen-induced mitochondrial injury does not result from only a high oxygen environment.

Moderately damaged mitochondria are known to consume more oxygen than do intact mitochondria during state 4 respiration, in which oxygen consumption is uncoupled with oxidative phosphorylation and may be attributable to the generation of activated oxygen (Loschen et al., 1971; Bovet and Chance, 1973). On the other hand, the myocardium contains little SOD or catalase in comparison with other principal tissue (Hein et al., 1975; Thayer, 1977; Doroshow et al., 1980), and the antioxidant ability would be further reduced by ischemia (Majeska et al., 1978; Guarnieri et al., 1980). This may...
explain why the 40-minute ischemic mitochondria, which appeared to be moderately damaged, were more significantly affected by oxygen toxicity than the less-damaged nonischemic and severely damaged 90-minute ischemic mitochondria. The border zone of the infarct area, consisting of salvageable myocardium, must also be subjected to oxygen-induced injury. The deleterious effect of high concentrations of intramyocardial calcium and of the use of inotropic drugs in the post-perfusion period may be involved in oxygen-induced injury due to the enhancement of oxygen consumption.

Lack of the enzymatic defense of the heart mitochondria against oxygen toxicity led us to use SOD and catalase. These enzymes successfully inhibited the formation of hydroxyl radical, the reduction of coenzyme Q10, and the impairment of oxidative phosphorylation. However, administration of SOD or catalase alone is insufficient to prevent oxygen-induced injury (Fong et al., 1973; Nohi et al., 1981), because each enzyme is inactivated in the presence of superoxide anion and hydrogen peroxide (Bray and Cockle, 1974; Kono and Fridovich, 1982). Combined administration of both enzymes is considered to be an effective and feasible preventive measure against oxygen-induced mitochondrial injury. The enzymatic decomposition of superoxide anion and hydrogen peroxide fundamentally removes the sources of peroxidation, while antioxidants such as coenzyme Q10, α-tocopherol, and ascorbate indirectly remove reactive oxygen metabolites through the peroxidative reaction.

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