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# Ischemia-Reperfusion Injury in Cardiovascular Systems

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**Abbreviations**

| | |
|---|---|
| AXC, aortic cross-clamping | ROS, reactive oxygen species |
| CAT, catalase | Se, selenium |
| CuZnSOD, copper zinc SOD | SOD, superoxide dismutase |
| DMPO, 5,5'-dimethyl-1 pyroline-N-oxide | TG, transgenic mice |
| ESR, electron spin resonance | XO, xanthion oxidase |
| GPx, glutathione peroxide | XDH, xanthine dehydrogenase |
| GR, glutathione reductase | |
| Hsps, heat shock proteins | |
| I/R, ischemia-reperfusion | |
| MnSOD, manganese SOD | |
| NOS, nitric oxidase synthase | |

**Abstract**

Cardiovascular diseases are the most common cause of death in the US. Coronary heart disease is the leading cause of death from cardiovascular disease. A burst of reactive oxygen species (ROS) has been described with the first moment of reperfusion and is associated with injury. Lipid and protein oxidation during ischemia/reperfusion (I/R) results in oxidative stress and poor physiological recovery. In hearts, transient I/R induces necrosis and apoptosis, leading to myocardial dysfunction. In normal conditions, ROS is controlled by endogenous free radical scavengers such as SOD, GPx, and catalase. Xanthine oxidase (XO) has been proposed as a source of toxic oxygen metabolites during reperfusion [16].

**Introduction**

Free radicals are a relatively new concept in recent decades, however, it is now accepted that I/R-caused ROS will cause cell damage. ROS formed in the early stage of reperfusion can initiate lipid peroxidation, oxidize proteins and cause DNA strand breaks. ROS creations can be both protective and deleterious. This is because ROS can be a signal to induce stress responses that lead to cell survival or apoptosis. Superoxide and hydrogen peroxide are produced in regular, healthy tissues can active ROS signaling protection. However, ischemia-reperfusion interrupts the normal balance and hydroxyl radicals ($HO^{\bullet}$) can be produced via the Fenton reaction. Free

radical scavengers are indirect evidence of cell damage by ROS. When scavengers were administered prior to reperfusion, a reduction of injury can be observed compared to the case where scavengers administered after reperfusion. The evidence of I/R induced injuries have been reported in many organs, such as heart, kidney, liver, and intestine [2, 11, and 13].

## Ischemia-reperfusion injury

### A. Ischemia injury

Myocardial ischemia is associated with inflammation, cardiac dysfunction, and arrhythmias. It is agreed that myocardial cells reach the point of irreversible injury when the biochemical processes of energy production can no longer maintain cellular integrity. Then, following loss of cellular integrity, excessive numbers of activated neutrophils and macrophages play a critical role in inflammation. The recruitment and adhesion of leukocytes to endothelial cells is an early step in inflammation. Tissue MPO activity has been shown to be a reliable index of neutrophil infiltration [16].

Chronic dysfunction of the endothelium such as hypertension, atherosclerosis, and heart failure can be other risk factors of ischemia. High cholesterol intake and heavy smoking increase vascular endothelium ROS production, which decreases NO bioavailability and may convert the normal anti-inflammatory type to a proinflammatory phenotype. In addition, atherosclerosis is

associated with increased vascular superoxide generation. NADPH oxidase-derived superoxide production may also influence the development of atherosclerotic lesions. Studies on human coronary artery diseases have shown increased NADPH oxidase subunit expression with increases *in situ* superoxide generation in the area of injury [1, 9, and 13].

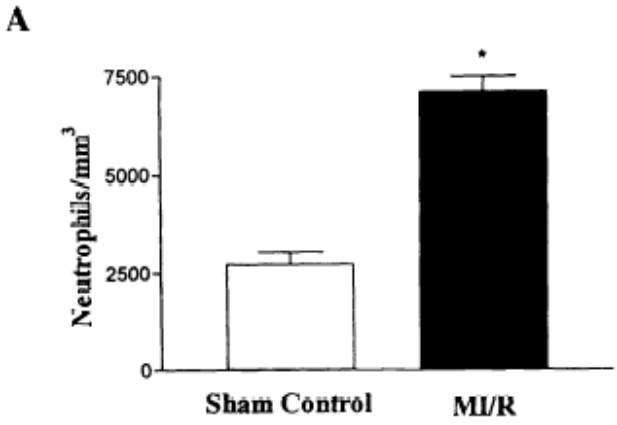


**Figure.1**

**Production of superoxide measured in IA (ischemia areas), NIA (nonischemic areas) of I/R and sham-operated animals. There was 140% increase of superoxide production in ischemic areas [13].**

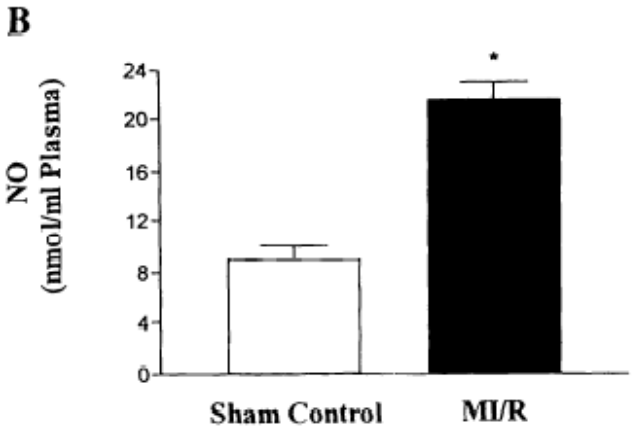


**Figure.2**

**Myocardial I/R induced a systemic inflammatory response.**
**(A) 160% increase in circulating neutrophils compared with sham-operated animals.**
**(B) Plasma NO as increased 140% after myocardial I/R compared with sham-operated animals [13].**

**B. Reperfusion injury**

Reperfusion of the ischemic myocardium must be performed to prevent glucose deprivation or necrosis. Unfortunately, the increased generation of ROS during reperfusion after ischemia contributes to issue injury. These ROS react with lipids, proteins, and DNA, resulting in altered cellular function. Many studies show that XO-mediated $O_2^{\bullet -}$ generation plays an important role in reperfusion injury. *Granger et al.* postulated that xanthine formed from the degradation of ATP during ischemia would be metabolized by xanthine oxidase to superoxide and uric acid following the reintroduction of oxygen during reperfusion. XO-derived $O_2^{\bullet -}$ can persist for several days or weeks after reperfusion. Myocardial I/R not only induces regional inflammatory responses but also induces systemic inflammatory responses, and manifests highly increased circulating neutrophils, NO, and $O_2^{\bullet -}$ compared with the sham-operated animals. Zweier *et al.* has identified a spectral peak of various free radicals in hearts during the first 10 s of reperfusion. Ambrosio *et al.* observed that both carbon- and oxygen-centered radicals occurred at 15-20 s following reperfusion [13, 16, and18].

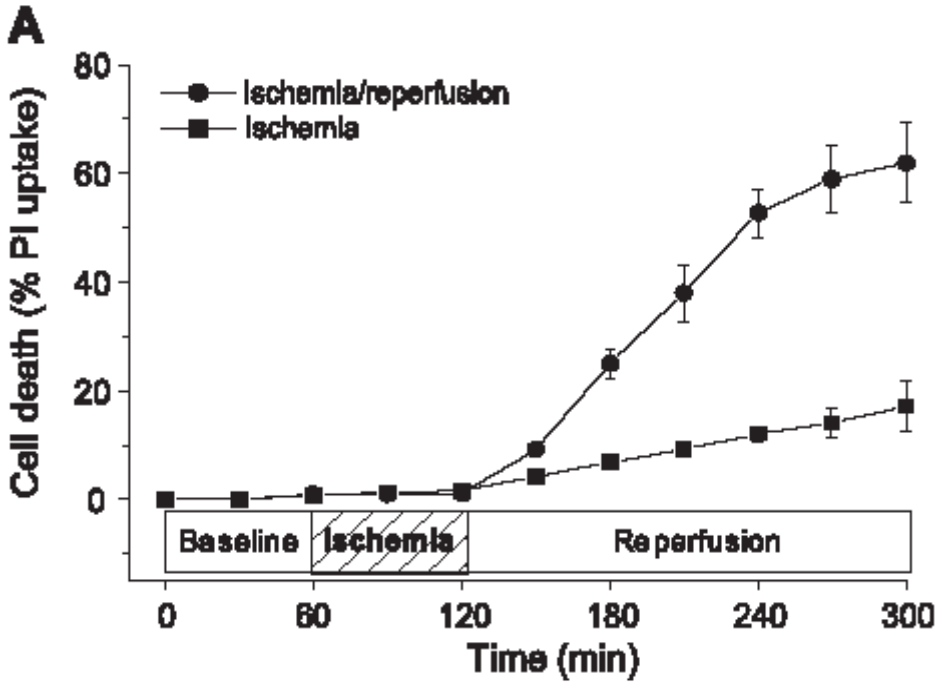


**Figure 3. Cardiomyocyte exposed to 1 h of ischemia followed by 3 h reperfusion demonstrate accelerated cell death during reperfusion, not during ischemia [16].**

**ROS generation and cardiovascular damage**

**A. Superoxide ($O_2^{\bullet -}$)**

The mitochondrial respiratory chain can be a major source of $O_2^{\bullet -}$. Superoxide production usually involves a one-electron reduction of molecular $O_2$ **(Reaction 2)**. The negative charge $O_2^{\bullet -}$ radical is unstable in aqueous solution and rapidly dismutates to $H_2O_2$. Superoxide dose not permeated the membrane efficiently. Superoxide reacts with NO at a faster rate than with SOD (rate constant~ $7 \times 10^9$ mol $\cdot$ $L^{-1} \cdot s^{-1}$) [1, 13, 18, and 21]. Therefore, NO can out compete SOD and react with $O_2^{\bullet -}$ to generate $ONOO^-$ **(Reaction 1)**. Peroxynitrite can activate metals that can

damage DNA molecules.

$O_2^{\bullet -} + NO \longrightarrow ONOO^- + H^+$ **[Reaction 1]**

**B. Hydrogen peroxide ($H_2O_2$)**

Mitochondria are key cellular sites for the production of hydrogen peroxide. Otherwise, mitochondrial density is high in heart cells, constituting up to 35% of the cell volume of rat heart muscle cells. High concentrations of hydrogen peroxide can represent a hazard to the cell, but in normal situations it also has a regulatory role in several processes, including signal transduction, cell development, cell proliferation, and apoptosis. Indeed, $H_2O_2$ is more stable and diffusible than $O_2^{\bullet -}$. It is also more cell membrane permeable therefore is more relevant than $O_2^{\bullet -}$ with respect to modulation of signal transduction [8 and 26]. The GPx-1 is the most important peroxidase for $H_2O_2$ removal in mammals because GPx-1 is present in cytosol, mitochondria, endoplasmic reticulum, and nuclei. Another enzyme to convert $H_2O_2$ to $H_2O$ is calatase (CAT), which is present in peroxisomes and at low levels in the cytosol and in heart mitochondria.

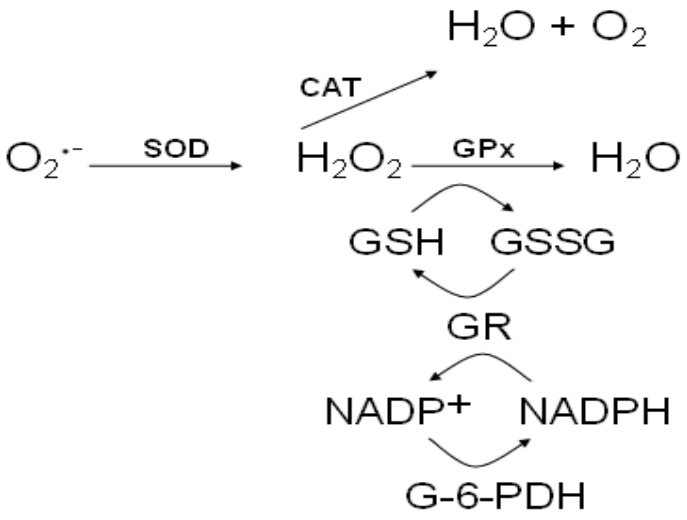


**Figure 4. SOD, CAT, and GPx antioxidant enzymes system.**

**C. Peroxynitrite ($ONOO^-$)**

Peroxynitrite ($ONOO^-$) is a biological oxidant from the reaction of $O_2^{\bullet -}$ and NO **(Reaction 1)**. Nitric oxide is generated by three isoforms of nitric oxide synthase (NOS), endothelial NOS (eNOS), inducible NOS (iNOS), and neuronal NOS (nNOS). Under normal conditiona, NO is primarily generated by eNOS in cardiac myocytes, and vascular endothelial cells. During the first seconds of reperfusion, the myocardium produces a surge of NO. At the same time, large amounts of $O_2^{\bullet -}$ are also generated. Nitric oxide and superoxide rapidly react during the early reperfusion to form peroxynitrite. NO is the only biological molecule known to compete with SOD for $O_2^{\bullet -}$. However, under normal conditions, the low amount of NO is not sufficient to compete effectively with SOD [2, 16, and 18].

**Evidence of ROS in Myocardial ischemia-reperfusion**

**A. Direct detection**

**Spin trapping**

ESR offers an indirect technique that provides the necessary evidence to confirm the involvement of these radicals. Carmen M. Arroyo *et al.* applied spin trapping to study free radical generation in ischemic and post-ischemia rat hearts. They detected a 5,5'-dimethyl-1-pyrroline-N-oxide (DMPO)-carbon radical and DMPO-OH adducts during ischemia. In post-ischemic myocardial reperfusion, they observed superoxide anion and hydroxyl radical. DMPO is a universal spin trap because of its low cytotoxicity, accessibility to the cell, and high rate constant of reaction with $HO^{\bullet}$ radicals. Purified DMPO solutions were used to test generation of $HO^{\bullet}$ and $O_2^{\bullet -}$ radicals. After 40 min of pre-ischemic perfusion in the presence of DMPO, no signal was observed [3].

$$2O_2^{\bullet -} + 2\,H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2 \qquad \textbf{[Reaction 2]}$$

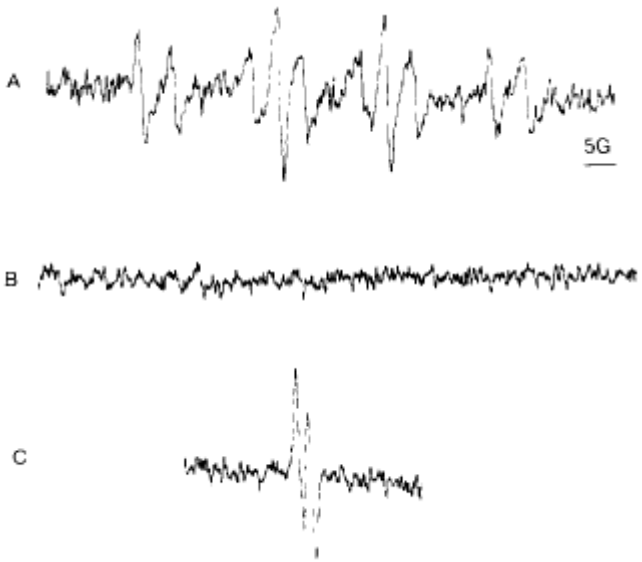


**Figure 5. ESR spectra obtained: (A) 10 min of ischemia without SOD. (B) 10 min of ischemia in the presence of SOD (93 ug/ml). (C) 30 min of ischemia in the presence of SOD [3].**

Their results demonstrated that short-lived free radicals are formed during myocardial ischemia and post-ischemic reperfusion. Superoxide production is also identified in the early phase of reperfusion. These studies also provide evidence that free radicals cause cell injury during ischemia-reperfusion [3 and 14].

## B. Indirect detection

### Antioxidants (To stop the propagation cycle)

Many studies have evaluated the influence of vitamin C, vitamin E and thiol compounds alone or in combination on ischemia-reperfusion. Dingchao *et al.* administrated high dose of ascorbic acid (205 mg/kg) in 85 patients undergoing cardiopulmonary bypass. They observed

that ascorbate-treated patients have higher postoperative index and shorter hospital stay than controls. However, Mickle and colleagues' studies with oral intake vitamin E faced difficulties in achieving myocardial concentration. They concluded that two weeks of oral vitamin E therapy was required to double the myocardial vitamin E concentration [4 and 17].

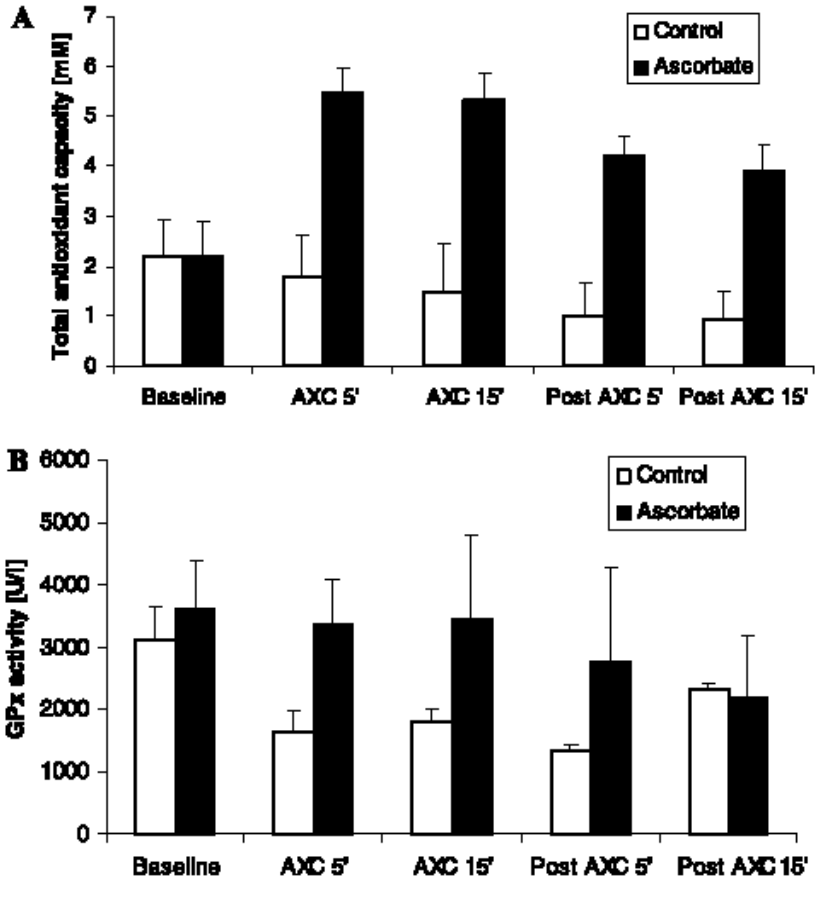


**Figure 6.**

**(A) Influence of high dose ascorbic acid administration on plasma redox properties [17].**

**(B)GPx activity in patents undergoing cardiac valve surgery and cardiopulmonary bypass with AXC. Treatment groups received 250 mg/kg total ascorbic acid in two equal doses before and after bypass and AXC. AXC (aortic cross-clamping) [17].**

**The mechanism of generation ROS in myocardium**

**A. XO in ischemia-reperfusion injury**

During ischemia, high concentration of hypoxanthine (HX) accumulates due to the degradation of ATP. Both XD and XO catalyze oxidation of hypoxanthine to xanthine and xanthine to urate. XO reduces $O_2$ to form $O_2^{\bullet -}$. XO-mediated ROS plays an important role in tissue injury during reperfusion. Cell culture studies about ROS-generating XO system indicate that $H_2O_2$ may be the key promoter of tissue injury in this setting. Another explanation of XO induced ROS is the accumulation and activation of neutrophils. Activation of neutrophils is part of the microvascular inflammatory response to pathogens. Otherwise, proinflammatory mediators can activate XO, such as TNF-$\alpha$, interleukins, and lipopolysaccharide [8, 13, 20, and 21].

Hypoxanthine + $O_2$ ⟶ Xanthine + $H_2O_2$ **[Reaction 3]**

Xanthine + $O_2$ + $2H_2O$ ⟶ Uric acid + $2H_2O_2$ **[Reaction 4]**

**Prevention of ROS damage**

**A. Endogenous antioxidants**

**a. MnSOD**

Manganese superoxide dismutase (MnSOD) is a key antioxidant that conducts superoxide

to hydrogen peroxide and oxygen **(reaction 2)**. Human MnSOD is a tetrameric enzyme with four identical subunits each harboring a $Mn^{3+}$ atom. MnSOD is a mitochondrial protein who primary functions is to protect mitochondria from superoxide. It is encoded in the nuclear chromatin, synthesized as a precursor in cytoplasm, and imported into mitochondrial matrix in its mature form [13, 17, 23, 26, 27, and 28].

**b. GPx**

The family of GPx comprises four distinct mammalian selenoproteins. The classical GPx-1 is ubiquitously distributed and also called cellular GPx. Selenium supplementation is used to promote the antioxidant activation GPx in myocardial function post-I/R. Glutathione peroxidases are homologous enzymes which share several common features such as catalyzed glutathione-dependent degradation of hydroperoxide and Se as a critical component of the enzyme active center. Many studies showed selenium deficiency down-regulates thioredoxin reductase and glutathione peroxidase activity, impairing the recovery of hearts from ischemia-reperfusion. K Venardos *et al*. showed rats fed a selenium-free diet exhibited poor functional recovery post-cardiac ischemia-reperfusion. Selenium supplement can be used to increase the systemic activity of both GPx and TrxR [8, 14, 15, 18,and 23].

GPx catalyses the reduction of peroxides using glutathione (GSH) as the reducing substrate

according the following equations:

$$ROOH + 2GSH \xrightarrow{GPx} ROH + GSSG + H_2O \quad \textbf{[Reaction 5]}$$

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2GSH + NADP^+ \quad \textbf{[Reaction 6]}$$

Follow the change in absorbance of NADPH, $A_{340} = 6200\ M^{-1}cm^{-1}$

| Selenoproteine | Function | Expression |
|---|---|---|
| Glutathione peroxidase 1 (cGPx) | Glutathion-dependent hydroperoxide removal | Ubiquitous |
| Glutathione peroxidase 2 (GPx-GI) | Glutathion-dependent Glutathion-dependent hydroperoxide removal | Gastrointestinal tract |
| Glutathione peroxidase 3 (pGPx) | Antioxidant (hydroperoxide removal) | Plasma |
| Glutathione peroxidase 4 (PHGPx) | Phospholipid hydroperoxide removal | Ubiquitous |

**Table 1. GPx family and their functions.**

**c. Hsps (Heat shock preteins)**

In the heart, ischemia-reperfusion induces necrosis and apoptosis, leading to myocardial dysfunction. It is known that Hsps synthesis is a tool to protect cells after exposure to heat or

deleterious stimuli. Hsps also is a mediator of myocardial protection, particularly in I/R injury. The cardioprotective effects of Hsp70 have been shown in isolated adult feline cardiomyocytes. Moreover, Hsp20, a small heat-shock protein, has been shown to regulate vasodilation and suppress platelet aggregration. Fan *et al.* indicated that Hsp20 improved contractile function and protected against $\beta$-agonist-mediated apoptosis. Transgenic mice models with overexpressed Hsp20 in the heart were protected against I/R injury. This implicates Hsp20 as a demanded therapeutic protein for ischemia disease [10].

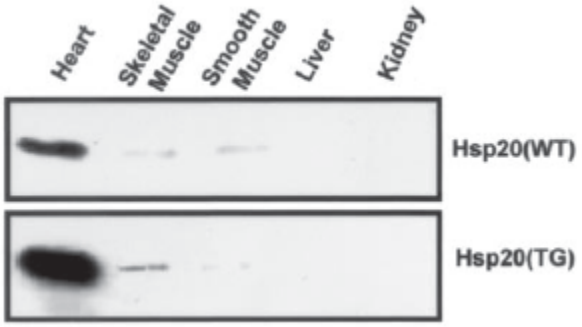


**Figure 7. Western blotting of wild type (WT) and Hsp20 transgenic mice (TG) of different tissue [10].**

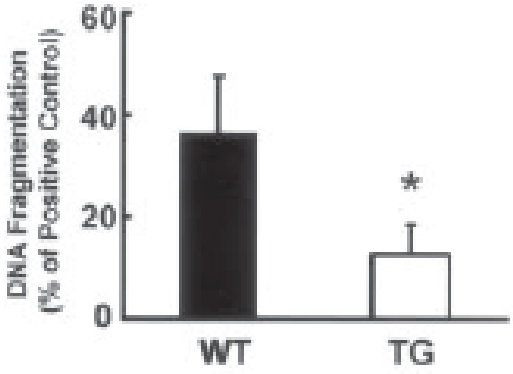


**Figure 8. DNA fragment ratio in WT is higher than TG [10].**

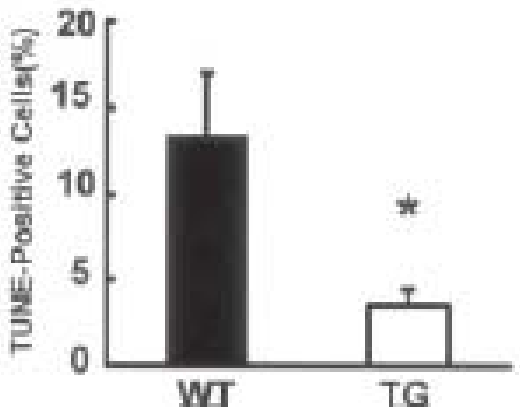


**Figure 9. WT has relatively high ratio of TUNEL-stain positive cells [10].**

**B. Exogenous antioxidants**

**a. NO**

The primary source of NO in biological tissues is the metabolism of L-arginine by NO synthase (NOS). But the generation of NO from L-arginine by NOS enzymes depends on oxygen, which is rapidly decreased in ischemia.

Ferrari *et al.* and Gabel *et al.* suggest that the reduction of $NO_2^-$ to NO is derived from a simple acidification reaction. The ischemia-induced acidosis in the isolated rat heart is lowered to about pH 5.5 after 20 min of global ischemia. In acidic conditions, $NO_2^-$ can chemically reduce back to NO. However, new data show that the reduction of $NO_2^-$ to NO needs catalysis by enzyme. Purified xanthine oxidoreductase (XOR), under hypoxia conditions, catalyzes the reduction of $NO_2^-$ to NO [1, 6, 16, and 18].

**b. Vit. C**

Humans don't have the ability to make our own vitamin C. We must obtain vitamin C through our diet. Vitamin C, also known as ascorbic acid, is a water-soluble antioxidant which is essential for normal functioning of the body **(Reaction 7)**. Dr Linus Pauling, father of vitamin C, declared that large intakes of vitamin C each day aids anti-cancer activity within the body. Ascorbate behaves as a weak singlet oxygen quencher, it is, in fact, a better one-electron reductant than tocopherol ($E^{\circ\prime}$=+0.23 V), and is supposed to recycle the tocopheroxyl radical in vivo **(Reaction 8 and 9)**. $Asc^{\bullet -}$ can be recycled by enzyme systems [4 and 17].

$AscH^- + R^\bullet \longrightarrow Asc^{\bullet -} + R\text{-}H$ **[Reaction 7]**

**c. Vit. E**

Tocopherols, a lipid soluble antioxidant, mainly act as free radical chain-breaking antioxidants in liposomes and cellular membranes, but they also possess reactivity as singlet oxygen quenchers and in repairing free radical damages in proteins. The reducing properties of tocopherols are also matched by ascorbate, however the lipophilic nature of vitamin E provides it with access to lipid sites in membranes and lipoproteins, a level at which ascorbate cannot act. In the case of quinones bearing hydrophobic chains, as ubiquinone, the excited state would be

confined to the hydrophobic phase, and the lipidic nature of tocopherols would confer an important advantage. Furthermore, the cytotoxicity of ascorbic acid is oxygen-dependent and related to its autooxidation process. It is then particularly important to balance of the amounts of vitamin C, vitamin E, and carotenoids in the body, since they play an interesting synergic effect agains lipid peroxidation; for example, when vitamin E turns into tocopheroxyl radical, this reacts with vitamin C to restore tocopherol [17 and 24].

$LOO^{\bullet} + TOH \longrightarrow LOOH + TO^{\bullet}$ [Reaction 8]

$TO^{\bullet} + AscH^{-} \longrightarrow TOH + Asc^{\bullet -}$ **[Reaction 9]**

**Hypothesis**

**A. Appropriate antioxidants and free radical scavengers can improve recovery from ischemia-reperfusion**

Since formation of free radicals is the major source of myocardial reperfusion damage, the most important mrthod to protect myocardial cells is to get rid of ROS. Free radicals production begins immediately after the start of reperfusion, therefore, administrating high doses of diffusible antioxidants and keeping antioxidants in a higher concentration in your body is the best way to lower reperfusion damage. The role of antioxidants is not to prevent the initiation of

ROS, it is to block the propagation of ROS formation.

Another method is to administrate scavengers before reperfusion.

**Experimental design and methods:**

Four groups of cardiovascular ischemia mice, administrate one group for antioxidants, one for scavengers, one for antioxidants + scavengers, and one for control. After reperfusion, we can evaluate the ROS formation in each group by ESR. We also can observe the tissue damage degree by biopsy.

**B. SOD mimics and GPx mimics can promote ROS clearance**

SOD and GPx are two important enzymes to eliminate free radicals in our body. Many data show that overexpression MnSOD and GPx can lower ROS damage *in vivo*. However, we cannot predict when cardiovascular ischemia will happen and overexpression also requires time. Ischemia in cardiovascular systems is an emergency situation, and it produces huge amounts of ROS at the beginning of reperfusion. That is why I don't believe overexpression of MnSOD or GPx will work on cardiovascular ischemia. Investigation of GPx mimics and SOD mimics is a good idea to improve resistance I/R injury. Administrating GPx mimics and SOD mimics is easier to control than overexpressing GPx and SOD in cells. In addition, SOD mimics and GPx mimics have several characters such as, low molecular weight, high cell permeability, stable, and

water solubility that are highly correlated to the enzyme function.

**Summary**

Like any muscle, the heart needs a constant supply of oxygen and nutrients that are carried to it by the blood in the coronary arteries. When the coronary arteries become narrowed or clogged and cannot supply enough blood to the heart, the result is coronary heart disease. Heart attack and stroke are common results of conditions that restrict or stop the blood flow to the heart or brain. Since ischemia happened, reperfusion damage is hard to prevent. The only hope that we can do is to lower ROS production and reduce cell damage.

Many studies have shown the high efficacy of the antioxidants in limiting tissue damage and tissue death during I/R injury. There also have been numerous epidemiological studies suggesting a positive connection between exercise and heart health. However, in my opinion, a balanced diet, exercise regularly and take antioxidant vitamins daily are equally important to prevent ischemia injury. Risk factors of ischemic heart diseases, such as family history of heart attack, high blood pressure, smoking, diabetes, high fat diet, high blood cholesterol, and obesity can all precipitate ischemic heart disorders. The only uncontrollable factor is family history of heart diasease, in other words, that means to prevent ischemia is depending on correct life attitude. Stop smoking, eat a healthy diet, maintain a healthy weight, exercise as much as

possible, avoid excessive drinking, and see your doctor to control blood pressure, cholesterol, and diabetes are best way to aviod to prevent ischemic cardiomyopathy. When it comes to prevent ischemia-reperfusion injury in cardiovascular systems, the old adage “better late than never” has never been so true.

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