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REDUCTION OF COLD INJURY BY SUPEROXIDE DISMUTASE AND CATALASE

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The pathophysiology of cold injury was examined by cooling a hind leg of an anesthetized New Zealand white rabbit. A flow probe and a thermocouple were placed in the leg to be cooled to monitor the blood flow and tissue temperature. After baseline measurements, the leg was cooled with a freezing mixture up to 0°C, which was followed by rewarming. The other leg served as control. In the experimental group, liposome-bound superoxide dismutase and catalase were infused through the femoral vein 15 minutes prior to putting the freezing mixture on the leg. Salicylic acid was injected through the femoral vein at the end of some experiments to assay hydroxy radical ($OH^{\cdot}$). Our results demonstrated reduction of local blood flow in cold-exposed leg, indicating development of ischemia. Creatine kinase and lactage dehydrogenase were increased during rewarming in conjunction with hydroxyl radical formation, phospholipid breakdown, and lipid peroxidation. Treatment with superoxide dismutase and catalase reduced $OH^{\cdot}$ formation, prevented phospholipid degradation, and decreased creatine kinase, lactate dehydrogenase, and malonaldehyde formation. These results indicate that rewarming of cooled tissue is associated with "rewarming injury" similar to "reperfusion injury", and that oxygen-derived free radicals play a significant role in the pathophysiology of such injury.

KEY WORDS: Superoxide dismutase, catalase, cold injury, free radicals, ischemia, reperfusion injury, rewarming injury.

INTRODUCTION

A tissue or cell, if exposed to a cold environment for a prolonged period of time, may be severely damaged.[1,2] In addition to cells being hyperosmolar and frozen, they are also subjected to ischemic insult during cold exposure because of the shifting of hemoglobin dissociation curve to the left, thereby preventing O_2 release to the tissues.[3,4]

Unless a tissue has already gone through the fourth degree of injury leading to arteriolar spasm and necrosis, blood flow is usually reinstituted during rewarming. Thus, the cold-injured tissues undergo two distinct phases: ischemia and reperfusion. Recent studies have indicated that reperfusion of ischemic tissue is subjected to so-called "reperfusion injury" which amplifies the ischemic injury.[5,6] This study was undertaken to examine whether cold-exposed tissues are also subjected to such reperfusion injury.

A growing body of evidence supports a significant role of oxygen-derived free radicals in the pathophysiology of reperfusion injury.[7-9] Therefore, we attempted to examine the presence of these free radicals in tissues during rewarming of cold tissue.

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Our studies indicated the presence of hydroxyl radical ($OH^{\cdot}$) in the tissue in conjunction with evidence of reperfusion injury. The combined treatment of superoxide dismutase (SOD) and catalase prevented cellular injury during rewarming.

MATERIALS AND METHODS

Animal Preparation

New Zealand white rabbits of about 2.5 kg body weight were anesthetized with xylazine and ketamine and maintained under anesthesia during the entire experiment. A tracheostomy was performed, and the rabbits were ventilated by a harvard ventilator. A flow probe was placed around the femoral artery, which was connected to a flow meter, which in turn was connected to a six-channel simultrace chart recorder. A continuous display of electrocardiogram (EKG) was obtained on Lead II by connecting the limb leads to the same recorder. One of the legs was then cooled down to 0°C with a freezing mixture containing ice and salt. Continuous monitoring of the interstitial temperature was achieved by inserting a thermocouple probe into the limb. Tissue temperature was maintained for 20 min at 0°C. The ice was then removed, and the limb was allowed to rewarm to room temperature. Another group of animals underwent an identical experimental protocol, except that they were treated either with lipose-bouned SOD plus catalase or liposome only through the femoral vein 15 min prior to cooling the leg. During the experiment, blood samples were withdrawn at regular intervals of time for the subsequent assay of CK, LDH, and MDA formation. Blood flow was also continuously monitored. At the end of the experiment, salicylate (2 mM) was injected through the femoral artery to trap free radical. The rabbits were immediately sacrificed by an overdose of sodium pentobarbital. Tissue biopsies were withdrawn for assay of $OH^{\cdot}$.

Preparation of Liposome-Bound SOD and Catalase

SOD and catalase were incorporated into liposomes by soniceting an equal volume of a mixture of phosphatidylcholine and either SOD or catalase in an ice-cold bath for 10 min. The lipose-bound SOD and catalase were seaprated from free SOD and catalase by employing Sephadex G-50 gel filtration.

Assay for $OH^{\cdot}$

The method used to trap and quantitate $OH^{\cdot}$ was similar to that described by Grootveld and Halliwell.[10] The tissue was homogenized under liquid N_2. The ground tissue was suspended in a buffer containing a mixture of sodium citrate (0.05 M) and sodium acetate (0.03 M) (pH 4.5); 50 μl of 70% perchloric acid was then added to the mixture. The resultant mixture was degassed and filtered through a Rainin Nylon-66 membrane filter (0.45 μM). The sample (20 μl) was injected onto an Altex Ultrasphere 3 μODs (75 × 4.6 mm) equipped in a Water Associates HPLC unit consisting of a Model 510 pump and a Model 460 electrochemical detector. The hydroxylated products of salicylic acid were eluted with buffer (degassed and filtered) containing 0.03 M sodium acetate and 0.05 M sodium citrate (pH 4.5) at a flow rate of 0.8 ml/min. The detector potential was maintained at 0.6 V, employing Ag/AgCl reference electrode.

Measurement of Lipid Peroxidation

Malonaldehyde was measured as an index for lipid peroxidation. Plasma (0.5 ml) was added to 0.5 ml ice-cold perchloric acid (15%) and then treated with 0.75% thiobarbituric acid (TBA) as described previously.[11] Samples were boiled for 20 min and centrifuged to remove the pellet. The color of the supernatant was read at 535 nm. The concentration of MDA (nmol/ml) was calculated by using a molar extinction coefficient of 156 mM^{-1} cm^{-1}.

Assay for CK and LDH

CK and LDH were assayed in plasma samples obtained from the femoral artery, using an assay kit obtained from Sigma Chemical Company (St. Louis, MO) as described elsewhere.[12]

Measurements of Phospholipids

At the end of each experiment, tissue biopsies were frozen in liquid nitrogen. Lipids were extracted with chloroform-methanol mixture by the method of Folch *et al.* Phospholipids, except for lysophosphatidylcholine (LPC), were separated on silica K6 plates (Whatman, Clifton, NJ) using a mixture of chloroforma-methanol-petroleum ether-acetic acid-boric acid (40:20:30:10:1.8, vol/vol/vol/vol/wt) as a solvent system.[13] LPC was separated on silica gel H plates (Analtech, Newark, DE) using a mixture of chloroform-methanol-acetic acid-water (75:25:3:4, vol/vol/vol/vol) as a solvent system. Neutral lipids were separated on silica GF plates (Analtech) using a mixture of hexane-diethyl ether-acetic acid (70:30:1, vol/vol/vol) as a developing solvent. The lipids on the silica gel plates were identified by cochromatography with authentic lipid standards after brief exposure with iodine vapor, scraped off, and quantitated by the method of Bartlett.[14]

Statistical Analysis

All measurements are expressed as mean values $\pm$ SEM. Student's t-test or two-tailed t-test was used for comparison of the data between two groups or within each group for each variable. For multigroup comparisons, analysis of variance was used. For those variable and time points at which the groups were not equal, multiple comparison methods were used to establish all possible pair-wise comparisons to better identify which groups differed from the others. Differences were considered significant when the p value was less than 0.05. Each point shown in the figures is the mean value of at least six different experiments in each group.

RESULTS

Monitoring of Heart by EKG

The electrocardiographic pattern showed regularity in rate and rhythm of heart during the entire period of cooling the leg from 28°C to 0°C. Heart rate was always maintained in steady condition even during rewarming after keeping the limb at 0°C for 20 min. There was no difference in heart rates among the control, liposome, and SOD-catalase groups.

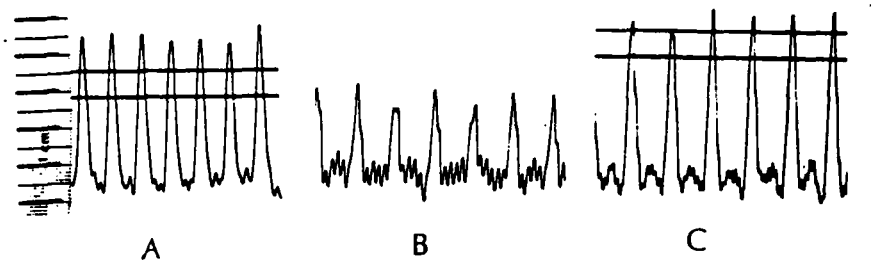

FIGURE 1 Changes in the pattern of blood flow waves in the femoral artery of rabbit leg using an ultrasonic flow probe. (A) Under normal conditions at room temperature. (B) During cooling of the leg at 0°C. (C) During rewarming period at room temperature.

Blood Flow During Cooling and Rewarming

Hemodynamic changes in the femoral artery were continuously monitored with the flow probe. The flow pattern was also recorded during the experiment (Figure 1). Under normal conditions, the flow rate was about 3.5 ml/min. During cooling of the limb, the flow rate continuously dropped. By the time the interstitial temperature became 0°C, it decreased to about 28% of the normal value to 1 ml/min (Figure 2). No difference was found in any of our results between the control and liposome groups, and hence we have used a liposome group as control in all of our results. A steady low flow rate was maintained for the rest of the cooling period. When rewarming was initiated by removing the freezing mixture from the leg, the flow rate gradually increased even when the interstitial temperature remained at 0°C. The flow rate

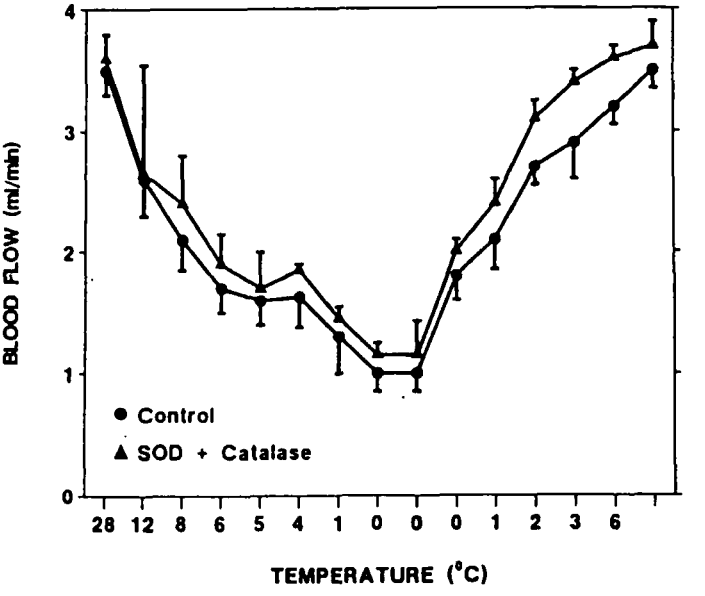

FIGURE 2 Effects of SOD plus catalase on the changes in the blood flow rate (ml/min) in the femoral artery of rabbit leg during cooling and rewarming. Blood flow was recorded using an ultrasonic flow probe. (O—O) control; (●—●) SOD + catalase.

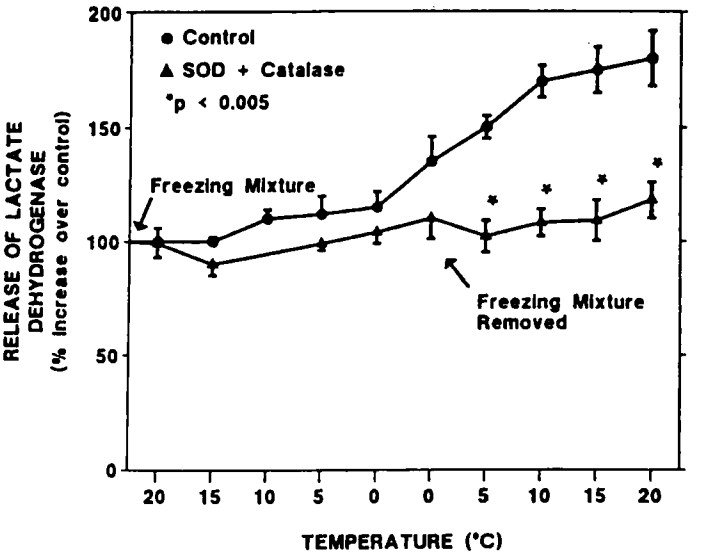

FIGURE 3 Effects of SOD plus catalase on the changes in the level of lactate dehydrogenase in plasma obtained from the femoral artery of rabbit leg during cooling and rewarming. (○—○) control; (●—●) SOD + catalase.

continued to rise, and at the end of the rewarming the flow rate was completely restored. Slightly higher flow was observed during rewarming in the SOD plus catalase group, but the difference was not statistically significant.

Release of LDH and CK

The release of arterial plasma LDH is plotted against temperature during cooling and rewarming of the rabbit leg (Figure 3). There was no change in plasma LDH levels during cooling from 30°C to 15°C, but below that temperature there was a slight increase in LDH release. A remarkable increase in plasma LDH (about 2-fold) was noted at the end of rewarming, suggesting that tissue damage occurred mostly during the rewarming period. SOD plus catalase prevented this increase in LDH release significantly. CK, another marker for tissue necrosis, followed a similar pattern (Figure 4). After cooling, a slight increase in CK activity was noticed, but the differences were not statistically significant. During rewarming, however, CK increased dramatically. At the end of reperfusion, these values were 2.5-fold higher compared to the base line levels. Once again, the enhanced CK release was significantly blocked by SOD plus catalase.

Estimation of Lipid Peroxidation

Malonaldehyde formation, a presumptive marker for lipid peroxidation, remained unchanged during cooling (Figure 5). During rewarming, MDA formation increased significantly and reached a 1.4-fold higher value compared to control at the end of the rewarming phase. This suggests that lipid peroxidation occurred only during rewarm-

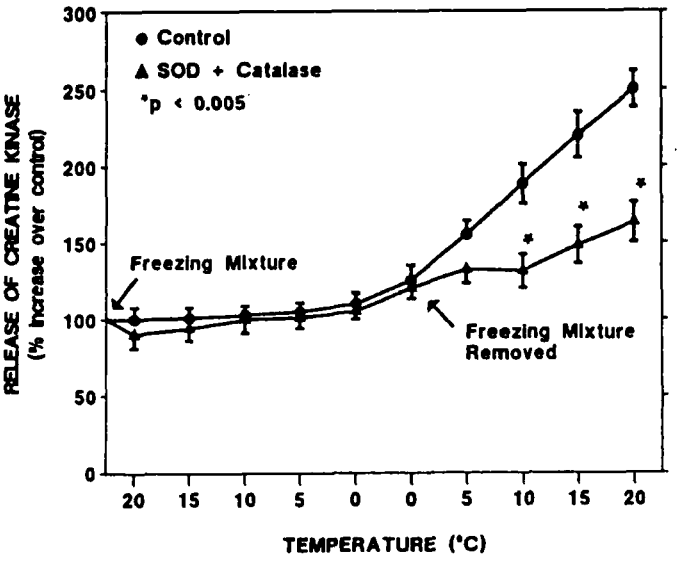

FIGURE 4 Effects of SOD plus catalase on the changes in the level of creatine kinase in plasma obtained from the femoral artery of rabbit leg during cooling and rewarming. (○—○) control; (●—●) SOD + catalase.

ing of the cooled tissue. The rise in MDA formation was minimal in the SOD plus catalase treated group.

Effects of Rewarming on Membrane Phospholipids

Table I shows the effect of rewarming on the contents of membrane phospholipids. There was a reduction in total phospholipids during rewarming compared to control, but the difference was not statistically significant. However, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) contents were decreased significantly, accompanied by increased LPC in the rewarmed tissue. The decrease in phospholipid content was inhibited by SOD and catalase treatment.

Estimation of Free Radical by HPLC

In some experiments, salicylate was injected through the femoral vein to trap any $OH^{\cdot}$ radical which might be generated in the tissue. We assayed the $OH^{\cdot}$ prior to as well as after cooling and at the onset of rewarming, when ice was removed from the leg and blood flow began to rise. We choose this point because oxygen-derived free radicals are known to be produced at the onset of reperfusion of an ischemic organ. The results are shown in Figure 6. The $OH^{\cdot}$ signal increased about 3-fold in the cooled tissue (after ice was removed, when blood flow began to rise, as shown in Figure 2) compared to baseline control values (Figure 6B compared to 6A). These signals were reduced significantly in the SOD plus catalase group, suggesting scavenging of $OH^{\cdot}$ radical.

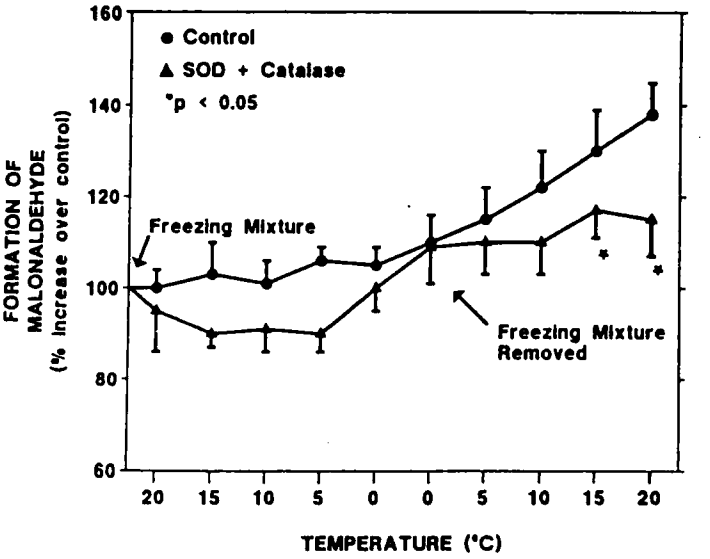

FIGURE 5 Effects of SOD plus catalase on the changes in the level of malonaldehyde in plasma obtained from the femoral artery of rabbit leg during cooling and rewarming. (O—O) control; (●—●) SOD + catalase.

DISCUSSION

There is increasing evbidence to indicate with a high degree of probability that endogenous free radical reactions play a significant role in the pathophysiology of reperfusion injury.[15-17] Our study indicates that cooling followed by rewarming undergoes similar pathophysiologic changes as those usually associated with the reperfusion of ischemic tissues. The cellular injury developed during cold exposure was amplified several-fold during rewarming, suggesting the occurrence of a phenomenon similar to that of reperfusion injury which is associated with reperfusion of ischemic tissues. This gives rise to a novel and interesting hypothesis concerning the pathophysiology of cold injury.

TABLE I
Effects of SOD plus catalase on the breakdown of membrane phospholipids in the cooled-rewarmed leg

	Control	SOD + catalase
Total phospholipids	8.29 ± 0.44	9.8 ± 0.80*
Phosphatidylcholine	4.23 ± 0.48	5.7 ± 0.74*
Phosphatidylethanolamine	1.9 ± 0.34	2.1 ± 0.28
Phosphatidylinositol	0.86 ± 0.13	1.0 ± 0.04
Lysophosphatidylcholine	0.07 ± 0.02	0.01 ± 0.01*

Rabbit leg was subjected to cold exposure and rewarming in the presence or absence of SOD and catalase, as shown in Figure 2. After the experiment, tissue biopsies were withdrawn, lipids extracted, and phospholipids separated and assayed as described in Methods. Resultes are expressed as Means ± SEM of six experiments in each group.

*$p < 0.05$ compared to control group.

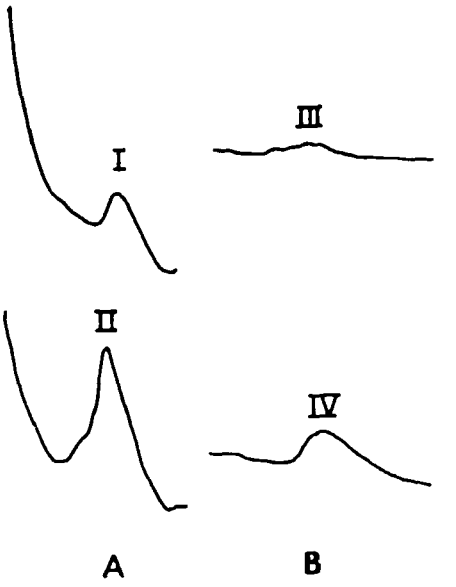

FIGURE 6 Chromatograms showing generation of $OH^{\cdot}$ in tissue biopsies obtained from leg. Salicylic acid was injected through the femoral artery, and then biopsies were withdrawn and immediately frozen under liquid nitrogen as described in Methods. Chromatograms were obtained with an HPLC using an electrochemical detector. I. Control tissue (pre-cooling). II. Cooled tissue at the onset of rewarming. III. SOD-treated control (pre-cooling). IV. SOD-treated cooled tissue at the onset of rewarming.

In the present study, we have clearly demonstrated that hypothermic insult resulted in reduction of blood flow, which dropped to 28% of the original value after cooling to 0°C. This value was almost restored after rewarming. We chose to hold the leg at 0°C up to a period of 20 min, because in this model, holding the temperature beyond 20 min resulted in only partial recovery of blood flow during rewarming. This suggests some irreversible injury during hypothermic insult. Indeed, it is known that poor perfusion in conjunction with anoxia and flow through cold-injured vessels can cause aggregation of red cells, platelets, and polymorphonuclear leukocytes (PMN) leading to thrombosis with tissue infarction, which ultimately may lead to gangrene formation.[18] In this study, free radical scavengers were unable to modify the blood flow in any way, suggesting that they did not play any role in recovering the blood flow in this model.

The presence of $OH^{\cdot}$ was conclusively demonstrated in the cooled-rewarmed tissue, indicating for the first time a role of oxygen-derived free radical in the pathophysiology of cold injury. The inhibition of $OH^{\cdot}$ formation by SOD and catalase treatment further confirms the development of oxygen-free radicals during rewarming. As described in this study, the HPLC technique to trap $OH^{\cdot}$ is a unique way to establish the presence of $OH^{\cdot}$. When salicylic acid is allowed to react with $OH^{\cdot}$, the hydroxylated products which are formed are extremely stable and can be detected by HPLC.[10] In order to demonstrate that the signal was indeed due to the formation of $OH^{\cdot}$, we used a specific $OH^{\cdot}$ scavenger such as dimethylsulfoxide (DMSO) to scavenge the $OH^{\cdot}$ signal. The presence of any $OH^{\cdot}$ signal described in this study was always confirmed by its scavenging with DMSO or SOD plus catalase.

The development of oxidative stress was further indicated by the presence of an increased amount of MDA formation, a presumptive marker for lipid peroxidation. It has been shown that oxygen-derived free radicals such as $OH^{\cdot}$ can attack the polyunstaurated fatty acids of membrane phospholipids, causing lipid peroxidation.[19] It is likely that the same is true in the present study. Indeed, our results have indicated degradation of membrane phospholipids, particularly PC and PE in the cooled-rewarmed tissue. The breakdown of membrane phospholipids was successfully inhibited by the free radical scavengers SOD plus catalase, suggesting that $OH^{\cdot}$ generated during the rewarming might be instrumental in the degradation of phospholipids and the development of lipid peroxidation.

The development of oxidative stress was accompanied by the increased release of LDH and CK, indicating increased cellular injury during rewarming. The reduction of LDH and CK release by SOD plus catalase once again suggests oxygen free radicals being a causative agent in the development of the tissue damage observed in our experiments.

In summary, our study has demonstrated for the first time the phenomenon of "rewarming injury" associated with the rewarming of cooled tissue. The pathophysiology of rewarming injury is similar to that of reperfusion injury developed during the revascularization of ischemic tissue. The simultaneous presence of $OH^{\cdot}$ and lipid peroxidation in conjunction with tissue injury and their inhibiton by SOD plus catalase suggests a role of oxygen derived free radicals in the development of "rewarming injury".

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References

1. M.H. Bourne, M.W. Piepkorn, F. Clayton, *et al.* (1986) Analysis of microvascular changes in frostbite injury. *Journal of Surgical Research*, **40**, 26–35.
2. W.D. Bowers, R.W. Habbard, R.C. Daum, *et al.* (1973) Ultrastructural studies of muscle cells and vascular endothelium immediately after freeze-thaw injury. *Cryobiology*, **10**, 9–21.
3. J.J. Gadarowski, Jr. and J.D. Esce (1984) Acute systemic change in blood cells, protein coagulation, fibrinolysis, and platelet aggregation after frostbite injury in the rabbit. *Cryobiology*, **21**, 359–370.
4. E. Mundth. Studies on the pathogenesis of cold injury. In *Proceedings of the Symposium on Arctic Medicine and Biology: IV Frostbite*, edited by E. Viereck, 1964. Fort Wainwright, AL: Arctic Aeromedical Laboratory.
5. H.J. Smith, K.M. Kent and S.E. Epstein (1978) Contractile damage from reperfusion after transient ischemia in the dog. *Journal of Thoracic and Cardiovascular Surgery*, **75**, 452–457.
6. D.K. Das, R.M. Engelman, J.A. Rousou, *et al.* (1986) Pathophysiology of superoxide radical as potential mediator of reperfusion injury in pig heart. *Basic Research in Cardiology*, **81**, 155–166.
7. D.A. Parks, G.B. Berkley, D.N. Granger, *et al.* (1982) Ischemic injury in the cat small intestine. Role of superoxide radicals. *Gastroenterology*, **82**, 9–15.
8. P.N. Manson, R.M. Anthelli, N.J. Im, *et al.* (1983) The role of oxygen-free radicals in ischemic tissue injury in island skin flaps. *Annals of Surgery*, **198**, 87–90.
9. S.R. Jolly, W.J. Kane, M.B. Bailie, *et al.* (1984) Canine myocardial reperfusion injury. Its reduction by combined administration of superoxide dismutase and catalase. *Circulation Research*, **54**, 277–285.
10. M. Grootveld and B. Halliwell (1986) Aromatic hydroxylation as a potential measure of hydroxyl radical formation *in vivo*. *Biochemical Journal*, **237**, 499–504.
11. M.R. Prasad, R. Clement, H. Otani, *et al.* (1988) Improved myocardial performance induced by

clofibrate during reperfusion after acute myocardial infarction. *Canadian Journal of Physiology and Pharmacology*,**66**, 1518–1523.
12. D.K. Das, D. Flansaas, R.M. Engelman, *et al.* (1987) Age related development profiles of the antioxidative system and the peroxidative status of the pig heart. *Biology of the Neonate*, **51**, 156–169.
13. H. Otani, M.R. Prasad, R.M. Jones, and Das, D.K. (1989) Mechanism of membrane phospholipid degradation in ischemic-reperfused rat hearts. *American Journal of Physiology*, **257**, H252–H258.
14. G.R. Barlett (1959) Phosphorous assay in column chromatography. *Journal of Biological Chemistry*, **234**, 466–468.
15. Y. Gauduel and M.A. Duvelleroy (1984) Role of oxygen radicals in cardiac injury due to reoxygenation. *Journal of Molecular and Cellular Cardiology*, **16**, 459–470.
16. H. Otani (1986) Cardiac performance during reperfusion improved by pretreatment with oxygen free radical scavengers. *Journal of Thoracic and Cardiovascular Surgery*, **91**, 290–295.
17. J. McCord (1985) Oxygen derived free radicals in postischemic tissue injury. *New England Journal of Medicine*, **312**, 159–163.
18. C.C. Bangs (1984) Hypothermia and frostbite. *Emergency Medicine Clinics of North America*, **2**, 475–487.
19. M.R. Prasad and D.K. Das (in press) Effect of oxygen free radicals on myocardial microsomal membrane integrity. *Free Radical Research Communications*.

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