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Age Related Differences in Oxygen Free Radical Injury during Myocardial Ischemia

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Abstract

(*J. Extra-Corpor. Technol.* 19[3] p. 245–257 Fall 1987, 42 ref.) Myocardial protection with an oxygenated cardioplegic solution was studied in mature and immature animals. Isolated adult and neonatal rabbit hearts were exposed to 90 minutes of hypothermic 30°C ischemia, and one of the following treatments: multidose oxygenated (pO_2>650 torr) cardioplegia, multidose nonoxygenated (pO_2<200 torr) cardioplegia, and a noncardioplegic Krebs-Henseleit solution. Upon reperfusion, adult hearts that had not received either cardioplegia treatment failed to recover postischemic hemodynamic function. However, neonates given the same treatment were able to recover 74.0 ± 2.4% (Mean ± SEM) of preischemic cardiac output and 69.2 ± 5.7% recovery of stroke work. Oxygenated cardioplegia administered to adult and neonatal hearts resulted in postischemic cardiac output recovery of 90.8 ± 2.4% and 86.5 ± 8.6%, respectively. Cardiac output recovery in nonoxygenated cardioplegia groups was 105.3 ± 3.9% in the adults, and 95.2 ± 6.6% in the neonates. Coronary sinus creatine kinase was substantially elevated in all adult groups, but not in the cardioplegia treated neonates. Membrane lipid peroxidation was assessed by measuring malondialdehyde in myocardial tissue homogenates. Malondialdehyde remained at control levels across all neonatal groups, but in the mature hearts the nonoxygenated cardioplegia group had significantly lower peroxidative products than the oxygenated hearts. The results of this study indicate an increased susceptibility of the mature myocardium to both the damaging effects of ischemia and free oxygen radicals. Administration of an oxygenated cardioplegic solution failed to provide adequate protection during moderate hypothermic arrest, and resulted in increased membrane peroxidative activity.

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Introduction

The formulation of cardioplegic solutions has long intrigued both the clinician and the basic scientist. The underlying premise of cardioplegia is to protect the ischemic myocardium by either enhancing metabolic function, or promoting an environment that minimizes the perturbations produced during interrupted flow. Many researchers have shown that the maintenance of aerobic metabolism during ischemia has the desired effect of generating high energy phosphate compounds, which help maintain cellular homeostasis.[1,2,3,4] Sanguineous cardioplegic solutions have increased oxygen carrying capacity, but may not provide optimum oxygen delivery. Increases in viscosity with decreasing temperature combined with alterations in oxyhemoglobin dissociation characteristics[5] may represent limitations in the use of blood cardioplegia. Improved myocardial protection by the hyperoxygenation of crystalloid cardioplegic solutions has been shown.[6,7] Several of the physical laws governing oxygen concentrations in crystalloid solutions include: increased oxygen solubility as temperature decreases, increased availability and utilization of oxygen, and substantially lower temperature related alterations in solution viscosity.[6]

Recently a group of highly toxic and reactive substances derived from molecular oxygen, free oxygen

radicals (FOR), have been shown to exacerbate myocardial ischemic injury.[8,9] FOR are thought to be involved in reperfusion injury mediated by the reintroduction of oxygen to ischemic myocardium.[10,11] It is known that the damaging consequences can be reduced when certain antioxidants are included in cardioplegic solutions.[12,13,14] However, the great majority of work has thus far been limited to effects on the mature myocardium, while physiologic and biochemical ontogenic differences are known to exist.

It is generally accepted that the neonatal myocardium tolerates hypoxia better than the mature heart,[15–18] yet elucidation of specific mechanisms remains obscure. Possible explanations include greater myocyte glycogen concentrations,[15] increased rates of glycolytic flux,[16,17] and increased fluidity of the sarcolemma.[18] Cellular membranes are particularly susceptible to the injurious effects of hypoxia. Hence, methods of maintaining their stability during ischemia greatly increases the cells' ability to recover. It has been shown that membrane characteristics vary with regard to developmental state, with differences existing in both the maintenance of calcium homeostasis,[19,20] and the distribution of certain membrane lipid components.[18,21] This study was designed to examine reperfusion/reoxygenation related effects of oxygenated and nonoxygenated cardioplegia in isolated neonatal and adult rabbit hearts. The specific indices include hemodynamic, metabolic, and cellular parameters of left ventricular function.

Materials and Methods

To study the effects of myocardial preservation an isolated working left heart preparation was utilized. Hearts were obtained from New Zealand White rabbits of either sex, and isolated in a similar fashion to that previously described.[22] Mature hearts were isolated from adult rabbits greater than 5 months of age, while neonatal hearts were obtained from 6-day-old to 8-day-old rabbits (Table 1). All animals received care according to the "Principles of Laboratory Animal Care" of the National Society of Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences (NIH Publication No. 80-23, revised 1978).

Hearts were randomly assigned into one of the following treatment groups: neonatal low dose oxygen cardioplegia (NLDO), neonatal high dose oxygen cardioplegia (NHDO), neonatal Krebs-Henseleit solution (NKH). Adult hearts received the same treatments and are designated as ALDO, AHDO, and AKH. LDO consisted of oxygen concentrations that reflect normal solubility at room temperature, generally having pO_2 levels less than 200 torr. HDO treatments included the oxygenation of the solutions to create pO_2 greater than 650 torr. Two additional groups consisting of adult and neonatal hearts were perfused only with KH buffer and, at the termination of ischemia, were immediately placed in liquid nitrogen and used as controls for biochemical analysis.

Table 1

Experimental Group Characteristics

Groups (n=6)	Age (days)	Rabbit Weight (Kg)	Heart Weight** (Gms)
NLDO	7.2±.5	0.14±.01	1.4±.0
NHDO	7.3±.3	0.13±.03	1.4±.1
NHK	7.2±.3	0.13±.01	1.3±.1
Control	7.3±.2	0.14±.01	1.0±.0
ALDO	*Adult	2.9±.1	11.1±.8
AHDO	*Adult	2.8±.2	10.3±.5
AKH	*Adult	2.8±.1	12.3±.6
Control	*Adult	2.9±.1	8.4±.3

NLDO—Neonate low dose oxygenated cardioplegia; NHDO—Neonate high dose oxygenated cardioplegia; NKH—Neonate Krebs-Henseleit; ALDO—Adult low dose oxygenated cardioplegia; AHDO—Adult high dose oxygenated cardioplegia; AKH—Adult Krebs-Henseleit.

*Greater than 22 weeks old; **Liquid nitrogen frozen weight.

Data are given as mean ± SEM

The experimental time course is listed in Figure 1. In the Langendorff mode[23] perfusion pressures in the neonatal groups were set at 55 cm H_2O, and adults at 100 cm H_2O, with the coronary sinus effluent discarded. After a 10 minute stabilization period the hearts were switched to the working heart mode with left

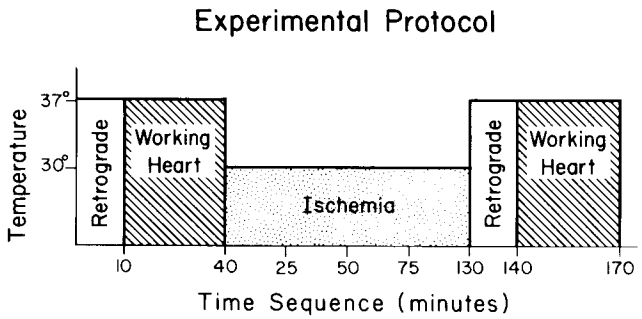

Figure 1: Experimental time course. Hemodynamic variables were measured both during the initial working heart period (preischemia), and during the postischemic working heart mode.

atrial filling pressures of 20 cm H_2O in the neonates and 25 cm H_2O in adults. Perfusate was spontaneously ejected from the left ventricle against an afterload equal to the initial perfusion pressures for each age class. During this period the stability of the heart was ascertained and preischemic baseline values were recorded. The heart was then transferred to a hypothermic (30°C) chamber and perfused with either a cardioplegic solution (CP) or KH at 30°C. CP was administered for 3 minutes during the onset of ischemia and every 25 minutes thereafter at perfusion pressures of 40 cm H_2O in the neonates and 75 cm H_2O in the adults. Hearts treated with KH buffer did not receive further washouts after their initial dose at the onset of ischemia. At the end of the ischemic interval the hearts were transferred back to the normothermic chamber and reperfused in the non-recirculating Langendorff manner for 10 minutes. The hearts were again placed in the working heart mode for an additional 30 minutes during which, hemodynamic measurements were recorded. At the termination of the experiment the hearts were immediately frozen in liquid nitrogen, weighed, and stored at −70°C.

Cardioplegia and Krebs-Henseleit perfusate compositions are listed in Table 2. Both HDO CP and KH were equilibrated with 95% O_2 and 5% CO_2 through a sintered glass filter to facilitate the dispersion of bubbles. All perfusates were double filtered through both qualitative filters and 50A 5 micron microfilters[a]. All solutions passed through inline bubble traps before entering the heart.

Hemodynamic and Metabolic Indices

The working heart model is essentially a left heart system with all hemodynamic parameters reflecting left ventricular function. Flows were measured during timed collections and included aortic flow, coronary flow, and cardiac output. Heart rate was recorded and stroke work (a measure of total left ventricular chamber function reflecting myocardial contractility) was calculated according to the following formula:

$$\text{Stroke work (Dynes cm}^{-1} \times 10^3) = \text{Aortic pressure (cm } H_2O) \times \text{Cardiac output (ml min}^{-1})/\text{Heart rate (beats min}^{-1})$$

Peak aortic systolic pressure was measured from a side arm port off the aortic ejection line. Left ventricular pressure was measured by the insertion of a ultraminature pressure transducer[b], via a side arm of the left atrial inflow line. The signal was interfaced into a Hewlett Packard (HP) 8805D pressure conditioner[c], and an HP 8805B medium gain amplifier for the determination of the rise in left ventricular pressure in respect to time (dP/dt).

Coronary sinus effluent samples were collected on ice at various times and assayed for creatine kinase (CK)[d], an enzymatic indicator of cellular damage. Additional samples were collected in 7% perchloric acid for the determination of lactic acid[e], an end product of glycolysis, which is known to accumulate in the absence of oxygen. Perfusate samples were collected during ischemia from the cardioplegic washouts and analyzed for pH, pO_2, and pCO_2[f]. Myocardial oxygen consumption (MVO_2) was determined from the following formula:

$$MVO_2 \text{ (ml of } O_2 \text{ gm}^{-1} \text{ min}^{-1}) = \text{Coronary blood flow (ml min}^{-1}) \times K \times (\text{Cardioplegic } pO_2 - \text{Venous } pO_2)$$

The constant K is equal to the O_2 solubility coefficient for crystalloid perfusate; 3.42×10^{-5} ml O_2 ml^{-1} of perfusate $mmHg^{-1}$ pO_2.[24] It is important to note that since an asanguineous perfusate was used, O_2 gas tensions were directly proportional to O_2 content, so that correction for O_2 saturation by hemoglobin was unnecessary.

Biochemical Indices

The frozen hearts were thawed gradually on ice in 4°C normal saline solution. Whole heart homogenates were prepared by use of a teflon homogenator in ice cold 1.5% KCl solution in a ratio of 9 ml gm^{-1} heart tissue. The homogenate was left for an hour on ice

Table 2
Perfusate Compositions

	Krebs-Henseleit	St. Thomas' Cardioplegia
NaCl (mmol·L^{-1})	120	110
KCl (mmol·L^{-1})	4.7	16
$CaCl_2$ (mmol·L^{-1})	2.5	1.2
$MgSO_4$ (mmol·L^{-1})	1.2	—
$MgCl_2$ (mmol·L^{-1})	—	16
KH_2PO_4 (mmol·L^{-1})	1.2	—
$NaHCO_3$ (mmol·L^{-1})	25	10
Glucose (mmol·L^{-1})	11.1	—
pH	7.4	7.9
(Osmolality mOsm·L^{-1}) calculated	323	324

a Gelman Sciences, Inc., Ann Arbor, MI 48106

b PR-249, Millar Instruments, Houston, TX 77252
c Hewlett-Packard Co, Andover, MA 01810
d Sigma kit CK No.47-UV and
e Sigma kit Lactate No.726-UV, Sigma Diagnostics, Inc., St. Louis, MO 63166
f Radiometer ABL II C, Radiometer Inc., Copenhagen, Denmark

and the supernatant transferred into separate culture tubes for analysis. Malondialdehyde, a fragmentation product formed by the oxidation of polyunsaturated fatty acids containing three or more double bonds, reacts colorimetrically with thiobarbituric acid and was assayed according to Ohkawa et al.[25] Protein was determined according to Lowry.[26] MDA is expressed in nmol MDA mg^{-1} of protein.

Statistical Analysis

All results are expressed as means ± standard error of the mean (SEM). All data were analyzed with the statistical program ABstat[g] and the use of an IBM PC microprocessor. "Student's paired t test" was utilized only to compare values within the same group. Percent recovery for hemodynamic parameters was determined for each heart, and the mean and SEM calculated for each group. Sample means were compared among groups by the use of either a one way or two way ANOVA. When significant the multiple comparison test Neuman-Keuls was performed to determine differences among groups. Dunnett's two-tailed test of significance was used to compare baseline values (preischemia) to multiple postischemic values.[27] Statistical significance was accepted at the $p<0.05$ level.

Results

Hemodynamic Recovery

Hemodynamic recovery is presented in Tables 3–6. Following ischemia hearts in the AKH group could not be weaned from the non-working Langendorff mode, and hence failed to recover. None of the AKH hearts were able to generate a pressure necessary to overcome the 100 cm H_2O afterload. In contrast both ALDO CP and ADHO CP recovered 101.2 ± 5% and 89.5 ± 4.5 (Figure 2) of their respective preischemic aortic flow (p = ns). Cardiac output (CO) recovered to 105.3 ± 3.9% in the ALDO group, while AHDO hearts recovered 90.8 ± 2.4% ($p<.01$). The trend in recovery for both CP adult groups was similar, with the LDO treated hearts displaying better overall return to preischemic values than HDO (Table 3). Intragroup comparison of preischemic to 30 minute postischemic values reflected no decline in function in the ALDO class. However, aortic flow, coronary flow and cardiac output all declined significantly in the AHDO (Table 4).

In the neonates all groups recovered following ischemia, although the NKH treated hearts had substantially lower postischemic values than either CP group (Table 5). The neonates exhibited a similar trend to that present in the adult hearts, namely, lower percent recovery in the NHDO group than the NLDO hearts. Recovery of aortic flow was greatest in NLDO 89.8 ± 7.2%, while the NHDO recovered 79.3 ± 9.3% (p = ns), and NKH only 60.5 ± 3.5% ($p<.05$ vs NLDO). Of interest in neonatal recovery was the precipitous increase in coronary flow seen across all groups. The KH treated hearts had the greatest percent increase in coronary flow (141.7 ± 6.6%) (Figure 3), and the lowest recovery of aortic flow, cardiac output and stroke work. Myocardial relaxation is expressed by the parameter − dP/dt which is a measure of left ventricular pressure decline. − dP/dt was depressed across all groups, but only reached significance in the NHDO treated hearts (Table 6).

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Table 3

Percent Recovery of Adult Preischemic Control Values

	ALDO Group 4	AHDO Group 5	AKH Group 6	P Value 4 vs 5	4 vs 6	5 vs 6
Aortic Flow	101.2 ± 8.5	89.5 ± 4.5	0.0 ± 0	NS	<.001	<.001
Coronary Flow	109.2 ± 5.2	91.2 ± 3.5	0.0 ± 0	<.025	<.001	<.001
Cardiac Output	105.3 ± 3.9	90.8 ± 2.4	0.0 ± 0	<.01	<.001	<.001
Stroke Work	125.8 ± 18.3	103.0 ± 9.3	0.0 ± 0	NS	<.001	<.001
Heart Rate	102.7 ± 7.3	93.2 ± 4.9	0.0 ± 0	NS	<.001	<.001
Peak LVP	108.6 ± 7.8	99.5 ± 2.2	0.0 ± 0	NS	<.001	<.001
Peak Aortic SP	116.8 ± 8.8	103.0 ± 3.8	0.0 ± 0	NS	<.001	<.001
+ dP/dt	107.1 ± 4.1	98.3 ± 3.4	0.0 ± 0	NS	<.001	<.001
− dP/dt	115.4 ± 11.4	102.8 ± 5.1	0.0 ± 0	NS	<.001	<.001

ALDO : Adult low dose oxygenated cardioplegia
AHDO : Adult high dose oxygenated cardioplegia
AKH : Adult Krebs-Henseleit
n = 6; Data are mean ± SEM

Table 4

Adult Hemodynamic Recovery

	ALDO			AHDO			AKH		
	Baseline	Postischemic	P Value	Baseline	Postischemic	P Value	Baseline	Postischemic	P Value
Aortic Flow ($ml \cdot min^{-1}$)	30.7 ± 2.3	30.7 ± 2.8	NS	32.3 ± 2.4	29.4 ± 3.4	<.05	28.6 ± 3.5	0.0 ± 0.0	<.001
Coronary Flow ($ml \cdot min^{-1}$)	32.5 ± 1.4	35.4 ± 2.2	NS	35.2 ± 1.6	31.9 ± 1.5	<.05	33.8 ± 2.8	0.0 ± 0.0	<.001
Cardiac Output ($ml \cdot min^{-1}$)	63.2 ± 3.0	66.1 ± 2.1	NS	67.5 ± 2.6	61.3 ± 3.1	<.05	62.3 ± 2.8	0.0 ± 0.0	<.001
Heart Rate ($beats \cdot min^{-1}$)	187.3 ± 10.9	190.0 ± 12.0	NS	189.3 ± 9.9	172.0 ± 9.9	NS	181.3 ± 5.0	0.0 ± 0.0	<.001
Stroke Work ($dynes \cdot cm^{-1} 10^3$)	38.0 ± 4.2	45.4 ± 4.7	NS	36.7 ± 5.1	36.8 ± 4.2	NS	41.1 ± 3.0	0.0 ± 0.0	<.001
Peak LVP (mmHg)	93.0 ± 3.3	100.3 ± 1.9	NS	97.8 ± 1.9	97.2 ± 3.0	NS	96.7 ± 1.8	0.0 ± 0.0	<.001
Peak Aortic SP (mmHg)	81.0 ± 4.1	86.2 ± 4.2	NS	91.8 ± 5.9	94.3 ± 5.8	NS	82.2 ± 4.3	0.0 ± 0.0	<.001
+ dP/dt ($mmHg \cdot sec^{-1}$)	1142 ± 121	1201 ± 107	NS	1243 ± 39	1221 ± 48	NS	1256 ± 36	0.0 ± 0.0	<.001
− dP/dt ($mmHg \cdot sec^{-1}$)	1071 ± 160	1150 ± 134	NS	1228 ± 50	1252 ± 29	NS	1150 ± 54	0.0 ± 0.0	<.001

ALDO: Adult low dose oxygenated cardioplegia
AHDO: Adult high dose oxygenated cardioplegia
LVP: Left ventricular pressure
Aortic SP: Aortic systolic pressure
n = 6 for each group; data are mean ± SEM

Table 5

Percent Recovery of Preischemic Control Values

	NLDO Group 1	NHDO Group 2	NKH Group 3	P Values 1 vs 2	1 vs 3	2 vs 3
Aortic Flow	89.8 ± 7.2	79.3 ± 9.3	60.5 ± 3.5	NS	<.05	NS
Coronary Flow	121.8 ± 9.1	113.7 ± 10.5	141.7 ± 6.6	NS	NS	NS
Cardiac Output	95.2 ± 6.6	86.5 ± 10.4	74.0 ± 2.4	NS	NS	NS
Stroke Work	93.2 ± 7.2	83.4 ± 10.4	69.2 ± 5.7	NS	NS	NS
Heart Rate	97.5 ± 5.5	101.3 ± 2.8	100.7 ± 4.7	NS	NS	NS
Peak LVP	95.7 ± 6.7	97.8 ± 2.6	88.7 ± 5.7	NS	NS	NS
Peak Aortic SP	98.2 ± 4.8	97.5 ± 5.5	92.5 ± 2.2	NS	NS	NS
+ dP/dt	96.0 ± 8.9	105.8 ± 11.4	96.2 ± 5.8	NS	NS	NS
− dP/dt	79.0 ± 13.7	77.7 ± 7.0	80.7 ± 8.0	NS	NS	NS

NLDO : Neonatal low dose oxygenated cardioplegia
NHDO: Neonatal high dose oxygenated cardioplegia
NKH : Neonatal Krebs-Henseleit
n = 6; data are mean ± SEM

Table 6

Neonatal Hemodynamic Recovery

	NLDO			NHDO			NKH		
	Baseline	Postischemic	P Value	Baseline	Postischemic	P Value	Baseline	Postischemic	P Value
Aortic Flow ($ml \cdot min^{-1}$)	19.8 ± 1.6	17.5 ± 1.3	NS	21.3 ± 1.8	16.8 ± 2.3	NS	20.6 ± 1.1	12.4 ± 0.9	<.001
Coronary Flow ($ml \cdot min^{-1}$)	4.6 ± 0.8	5.3 ± 0.7	NS	5.7 ± 0.9	6.3 ± 0.8	NS	4.0 ± 0.7	5.7 ± 1.2	<.05
Cardiac Output ($ml \cdot min^{-1}$)	24.3 ± 1.7	22.8 ± 1.5	NS	27.0 ± 2.0	23.0 ± 2.4	NS	24.6 ± 1.6	18.1 ± 1.4	<.001
Heart Rate ($beats \cdot min^{-1}$)	211.7 ± 7.8	206.0 ± 11.8	NS	229.3 ± 4.4	232.7 ± 7.8	NS	206.0 ± 13.6	206.7 ± 16.2	NS
Stroke Work ($dynes \cdot cm^{-1}10^3$)	9.1 ± 0.5	8.4 ± 0.9	NS	10.1 ± 1.1	8.2 ± 1.1	NS	11.1 ± 0.7	7.8 ± 0.9	<.005
Peak LVP (mmHg)	74.9 ± 4.4	70.9 ± 3.9	NS	86.7 ± 6.0	84.9 ± 6.5	NS	78.3 ± 1.8	69.3 ± 4.6	NS
Peak Aortic SP (mmHg)	58.2 ± 1.9	57.0 ± 3.4	NS	62.6 ± 3.4	59.9 ± 2.9	NS	68.3 ± 2.9	63.2 ± 2.6	<.05
+ dP/dt ($mmHg \cdot sec^{-1}$)	1071 ± 79	1004 ± 74	NS	1070 ± 106	1075 ± 56	NS	1192 ± 72	1129 ± 53	NS
− dP/dt ($mmHg \cdot sec^{-1}$)	867 ± 109	633 ± 77	NS	792 ± 50	604 ± 36	<.05	775 ± 42	629 ± 77	NS

NLDO: Neonatal low dose oxygenated cardioplegia
NHDO: Neonatal high dose oxygenated cardioplegia
LVP: Left ventricular pressure
Aortic SP: Aortic systolic pressure
n = 6 for each group; data are mean ± SEM

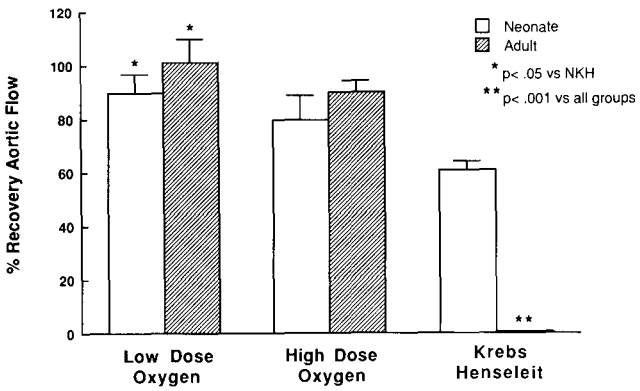

Figure 2: Percent recovery of preischemic aortic flow. NKH, neonate Krebs-Henseleit. n = 6 for each group. All data are mean ± SEM.

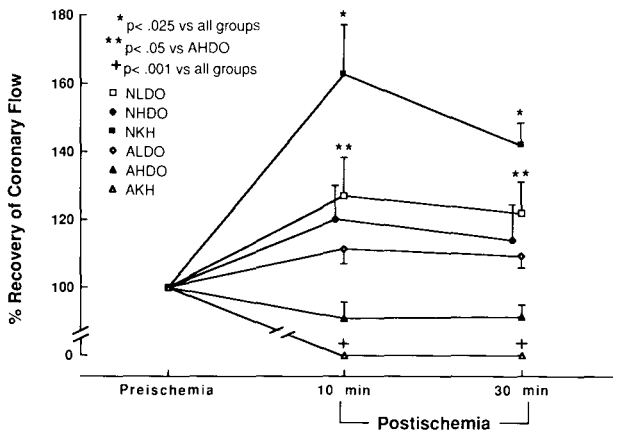

Figure 3: Percent recovery of preischemic coronary flow. NLDO, neonate low dose oxygen. NHDO, neonate high dose oxygen. NKH, neonate Krebs-Henseleit. ALDO, adult low dose oxygen. AHDO, adult high dose oxygen. AKH, adult Krebs-Henseleit. n = 6 for each group. All data are mean ± SEM.

Total coronary sinus flow was collected during the multidose administration of CP at each of the 3 minute washout periods (Table 7). At each of the 25, 50 and 75 minute collection times, both the adult and neonate LDO hearts had consistently lower coronary flows than did their HDO counterparts. When the total coronary washout during ischemia was calculated the trend was evident in lower volumes for the ALDO and NLDO groups, although significant differences were not met. Since the head pressure of CP delivery was maintained as a constant, the higher flows are a result of changes in coronary vascular resistance. It is important to note that oxygenated cardioplegia failed to induce rapid asystole during both the initial administration of cardioplegia and during subsequent doses, which may effect coronary flow. The summation of coronary flow during the preischemic working heart period was compared to that following ischemia. In the neonates, the LDO group total postischemic flow was elevated, but did not reach significance. NHDO increased from 152.4 ± 32.2 ml to 200.6 ± 33.3 ml following ischemia ($p<.01$), while NKH was elevated from 119.3 ± 36.8 ml to 183.2 ± 47.0 ml ($p<.01$).

MVO_2 was determined during the 50 minute multidose washout period and is expressed on a per gram of wet weight basis. MVO_2 was 0.39 ± 0.1 and 0.52 ± 0.1 ml O_2 gm^{-1} min^{-1} in the NLDO and ALDO groups, while the NHDO and AHDO groups consumed 5.27 ± 1.0 and 5.42 ± 0.7 ml O_2 gm^{-1} min^{-1}, respectively ($p<.001$ vs NLDO and ALDO). These substantial differences are in agreement with the increased delivery of O_2 with the oxygenated solutions, and help explain the observed tendency of both AHDO and NHDO to generate mechanical function during multidose reperfusion. Although high energy phosphates were not measured, the occurrence of spontaneous contractions supports the major benefit of enhancing aerobic metabolism in oxygenated groups, namely an increased production of adenosine triphosphate (ATP). The failure to maintain asystole during cardioplegia administration may not have the desired effect of providing an optimal cellular environment conducive to maximizing postischemic recovery. It is thought that if electromechanical function could be restricted in the early reperfusion period the increased production of ATP by aerobic metabolism could be channeled into reparative processes of various cellular fractions,[28,29] rather than wasting energy on unnecessary myocardial work. Perhaps the expected benefit of increased ATP production is negated when asystole is not adequately maintained during ischemia, and accents the role of hypothermia in cardioprotection.

Enzymatic and Metabolic Parameters

The release of creatine kinase (CK), an intracellular enzyme, into coronary sinus effluent has been used as an indicator of increased cellular permeability caused by the damaging effects of ischemia. Baseline values in the adult hearts were established during the preischemic period and were generally similar. The control group for biochemical analysis had slightly, but significantly, higher values than AKH treated hearts (Figure 4). CK values were elevated substantially during each of the washout periods in both ALDO and

Table 7

Total Coronary Flow Preischemia, Postischemia, and during Cardioplegic Washout

	NLDO	NHDO	NKH	ALDO	AHDO	AKH
Total Coronary Flow preischemic (ml)	133.40 ± 36.18	152.40* ± 32.17	119.33* ± 36.84	903.17 ± 46.59	1035.00 ± 43.59	968.33 ± 107.01
Total Coronary Flow postischemic (ml)	172.00 ± 27.92	200.60 ± 33.30	183.17 ± 47.04	1058.50 ± 63.92	821.00 ± 70.89	—**
3 min Washout 25 min	16.00 ± 3.16	17.75 ± 1.86	—	106.67 ± 7.48	127.67 ± 11.73	—
3 min Washout 50 min	13.75 ± 3.27	17.00 ± 2.52	—	118.00 ± 8.01	123.50 ± 9.87	—
3 min Washout 75 min	13.42 ± 3.31	16.58 ± 3.41	—	131.00 ± 8.12	142.67 ± 5.50	—**
Total Coronary Flow during ischemia	43.17 ± 9.04	51.33 ± 7.24	—	355.33 ± 22.08	393.83 ± 24.24	—**

NLDO—neonate low dose oxygenated cardioplegia; NHDO—neonate high dose oxygenated cardioplegia; NKH—neonate with oxygenated Krebs-Henseleit; ALDO—adult low dose oxygenated cardioplegia; AHDO—adult high dose oxygenated cardioplegia; AKH—adult Krebs-Henseleit

All data are mean ± SEM; n = 6 for each group.

*$p<.01$ vs postischemic flow; **no recovery

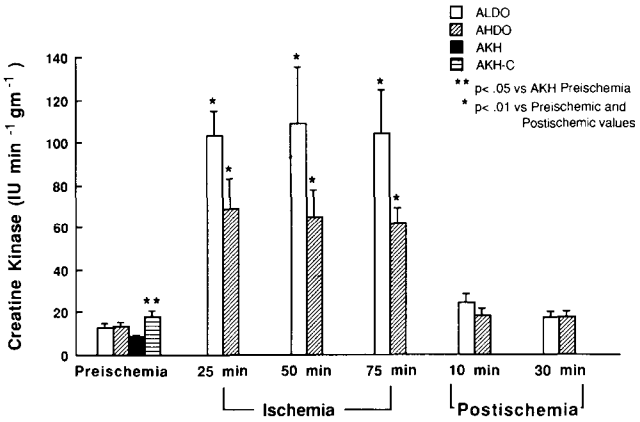

Figure 4: Adult heart coronary sinus creatine kinase release. ALDO, adult low dose oxygen. AHDO, adult high dose oxygen. AKH, adult Krebs-Henseleit. AKH-C, adult Krebs-Henseleit biochemical control. n = 6 for each group. All data are mean ± SEM.

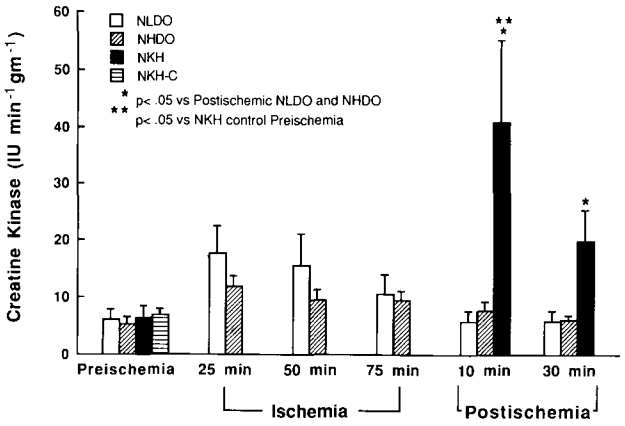

Figure 5: Neonatal coronary sinus creatine kinase release. NLDO, neonate low dose oxygen. NHDO, neonate high dose oxygen. NKH, neonate Krebs-Henseleit. NKH-C, neonate Krebs-Henseleit biochemical control. n = 6 for each group. All data are mean ± SEM.

with the greatest increase seen in the LDO group which increased from 12.7 ± 2.4 IU min^{-1} gm^{-1} to 109.0 ± 26.4 IU min^{-1} gm^{-1} ($p<.01$). During the reperfusion period, however, CK decreased in both groups to near baseline levels. In the neonates CK was also elevated during the ischemic period, but did not reach significant levels (Figure 5). Upon reperfusion CK declined to baseline levels in both groups receiving CP, but was substantially elevated in the NKH hearts.

Comparison of CK among groups, across time, revealed a significantly higher release in adult hearts compared to neonates. When expressed per gram of wet tissue weight, both adult groups consistently had greater levels of CK during ischemia than did either neonate group. During reperfusion, however, this effect was no longer evident and of those groups that were able to hemodynamically recover, the only significant increase was seen in the NKH treated hearts.

Lactate is an end product of the Embden-Meyerhoff pathway which in the presence of oxygen is metabolized to form the 2 carbon fragments of Acetyl CoA that enter the citric acid cycle. The concentration of lactate in coronary sinus samples of oxygenated isolated rabbit hearts is usually very low and difficult to quantify. However, in the presence of ischemia, lactate values increase tremendously due to the cessation of normal oxidative pathways. All multidose groups displayed significant increases in the production of lactate as measured in the washout volumes collected during CP administration (Table 8). An interesting trend was noticed within both oxygenated and non-oxygenated treatment groups. Both NLDO and ALDO exhibited a rapid rise in lactate release during ischemia that plateaued at the 25 minute washout, and remained at that level for the duration of the ischemic period. In the NHDO and AHDO groups, however, although a rapid rise in lactate was seen at the 25 minute washout time of ischemia, the ensuing 50 and 75 minute periods resulted in lesser quantities appearing in the coronary sinus effluent. This disparity in lactate accumulation supports the increased aerobiosis seen in oxygenated cardioplegic solutions compared to non-oxygenated. Upon reperfusion with KH buffer all groups returned to their preischemic baseline values.

Free oxygen radicals are known to participate in the propagation of membrane lipid peroxidation. Malondialdehyde (MDA) is released when polyunsaturated free fatty acids are oxidized, and can be correlated with membrane disruption and injury. Peroxidation injury caused by 90 minutes of ischemia without myocardial protection provided by CP was determined by assaying both neonatal and adult hearts frozen in liquid nitrogen without reperfusion (Figure 6). In the neonates, total MDA production was 2.2 ± .06 nmol mg^{-1} protein, while the same treatment in the adults resulted in 8.7 ± .17 nmol mg^{-1} protein. MDA release within the remaining neonatal groups was as follows: NLDO, 2.2 ± .05, NHDO, 3.6 ± .07, and NKH, 2.3 ± .04 nmol mg^{-1} protein (p = ns). In the adult groups the greatest increase in MDA production was seen in reperfused KH treated hearts with 10.2 ± .32 nmol MDA mg^{-1} protein. ALDO treated hearts had significantly lower levels of MDA, 2.5 ± .03 nmol MDA mg^{-1} protein, than AHDO hearts at 6.0 ± .14 nmol MDA mg^{-1} protein ($p<.05$).

Table 8

Lactate ($mmol \cdot L^{-1}$)

Groups	Preischemia Control	Ischemia 25 min	Ischemia 50 min	Ischemia 75 min	Postischemia 10 min	Postischemia 30 min
NLDO (n = 6)	.08 ± .03	.58* ± .09	.57* ± .11	.36 ± .11	.20 ± .09	.12 ± .04
NHDO (n = 6)	.03 ± .02	.83* ± .10	.28† ± .08	.34 ± .08	.05 ± .02	.04 ± .04
NKH (n = 6)	.13 ± .05	—	—	—	.11 ± .03	.15 ± .04
ALDO (n = 6)	.24 ± .04	.76* ± .09	.70* ± .08	.75* ± .12	.16 ± .04	.14 ± .03
AHDO (n = 6)	.15 ± .05	.90* ± .20	.48 ± .07	.49 ± .11	.13 ± .05	.16 ± .06
AKH (n = 6)	.07 ± .04	—	—	—	−Δ	−Δ

NLDO—neonatal low dose oxygenated cardioplegia; NHDO—neonatal high dose oxygenated cardioplegia; NKH—neonatal Krebs-Henseleit; ALDO—adult low dose oxygenated cardioplegia; AHDO—adult high dose oxygenated cardioplegia; AKH—adult Krebs-Henseleit

*$p < .01$ vs control; †$p < .05$ vs control; Δ no recovery

Data are mean ± SEM

Discussion

Cardioplegia is the routine method of myocardial preservation utilized within most cardiac centers throughout the world.[1] Its wide use may only be surpassed by the multitude of "recipes" used in formulation. Scientific analysis of the efficacy of protective agents has been at best confusing, and may be dependent both upon the model employed, and the indices chosen to reflect the results of the treatment. In several previous studies we have shown that the isolated working heart preparation is a stable method of detecting alterations in myocardial function derived from ischemia.[22,30] We initially determined that there exists a differential response to myocardial ischemia in both adult and neonatal rabbit hearts.[22] The excellent recovery seen in the newborn hearts, with protection afforded only by hypothermia (20°C) motivated us to explore the efficacy of cardioplegia in protecting the newborn heart. Both single and multidose cardioplegia provided superior protection when compared to Krebs-Henseleit treated hearts.[30] However, serendipitously we discovered that hearts treated with a multidose oxygenated noncardioplegic solution fared worse than did hearts protected with only a single washout. The present study was undertaken to examine the effects of increasing the tension of oxygen in crystalloid cardioplegic solutions used to protect mature and neonatal hearts.

All methods of myocardial preservation have as their underlying common denominator the goal of minimizing cellular damage caused by hypoxia. It is generally accepted that cellular homeostasis is best maintained when the energy supply and demand ratio is upheld, and ionic balances and associated control mechanisms kept intact. Aerobic metabolism greatly decreases during periods of ischemia due to the unavailability of the electron acceptor molecular oxygen, necessary for the production of high energy phosphates through oxidative phosphorylation. The shift to anaerobic metabolic pathways is accompanied with a tumultuous decline in ATP production, which may ultimately limit cellular recovery following ischemia. Advocates of sanguineous CP solutions argue the benefits of the inclusion of red blood cells, and ultimately hemoglobin, in delivering high quantities of O_2.[1,2,3]

In the same light, proponents of oxygenated asanguineous CP point to the increased content of O_2 versus nonoxygenated CP, as a means of stimulating aerobic metabolism.[6,7,31] High O_2 solubility emulsions, chemicals in the perfluorocarbon family, are capable of carrying greater quantities of molecular O_2 supporting increased delivery. The role of molecular O_2, however, has recently come under intense scrutiny because of its involvement in reperfusion related phenomena. Accordingly, reevaluation of its role in cellular protection should be accomplished.

During normal aerobic metabolism energy is produced in the form of molecular bonds from the electron donators nicotinamide adenine dinucleotide (NADH) and flavine adenine dinucleotide ($FADH_2$), produced in the citric acid cycle. These molecules donate their electrons to the acceptor O_2, which undergoes a tetravalent electron reduction generating H_2O. In the absence of O_2, ATP catabolism produces adenine nucleotides ADP and AMP. Further breakdown results in the formation of adenosine, inosine, and the free purine base hypoxanthine. During reoxygenation the conversion of xanthine dehydrogenase to xanthine oxidase promotes the univalent reduction of O_2 through

a pathway that produces several highly reactive and toxic intermediates called free oxygen radicals (FOR). These molecules participate in a series of reactions which exacerbate ischemic injury. It is beyond the intent of this paper to explore the wide range of alterations precipitated by FOR. Therefore, the reader is referred to several excellent reviews on the subject.[8,9,10,11]

FOR include superoxide anion (O_2-) hydrogen peroxide (H_2O_2), and hydroxyl radical (OH), with the latter being most reactive. These species are produced in the mitochondria and migrate to the cytosol and cellular membranes where they are involved in a series of reactions that damage the cell. Susceptible biomolecules include both transport proteins and lipid fractions of the various membrane components.[32,33] The phospholipids of the cell membranes contain polyunsaturated fatty acids (PUFA) that are easily peroxidized by FOR, producing fragmentation products which include MDA. MDA has been shown to interfere with ionic gradients and enzyme activity by altering membrane morphology.[9] Therefore, MDA has been used as an indicator for the oxidation of membrane PUFA which in turn reflects the extent of cellular damage.

In the present study the increased production of MDA in the AHDO signifies membrane perturbations that were substantially greater than the ALDO treated hearts. Indeed, membrane injury has been shown to be modulated by oxygen tensions in the perfusing media,[9] with high O_2 concentrations resulting in FOR production greater than the basal states.

Hearse et al. have shown that reoxygenation related cellular damage is proportional to both the period of hypoxia and the pO_2 level present in the reoxygenation medium.[34,35] The readmission of O_2 to hypoxic tissue results in increased PUFA peroxidation with increased membrane permeability and altered mitochondrial function.[36] The most pronounced increase in MDA release was seen in the adult nonreperfused KH group, indicating severe ischemic membrane injury. CP containing low levels of oxygen was shown to be an effective method of limiting membrane injury during reperfusion. Multiple doses of a hyperoxygenated CP solution, however, resulted in higher levels of peroxidation products that were not significantly lower than quantities present when hearts were not protected with CP.

Although the greatest release of MDA in the neonatal hearts was found in the HDO group, statistical significance was not met. The peroxidation of membrane lipids among the various newborn groups did not vary, and CP did not limit the overall production of MDA. This can best be explained by examining the lipid contents of membranes of both newborn and adult rabbit hearts. Nagatomo et al.[21,37] have shown that the total lipid content per mg of protein was substantially higher in the sarcolemma and sarcoplasmic reticulum of newborn hearts compared to adults. They have also shown that the levels of phospholipids and cholesterol of various microsomal fractions were higher in the newborns.[21] However, analysis of the fatty acid components of the phospholipid molecules in neonatal membranes showed a greater quantity of saturation of the acyl chains than present in the adults. The PUFA linolenic and arachiodonic acids were significantly lower in all newborn microsomal fractions than the adults. This suggests that the changes in fatty acid composition may be related to the alterations in physical state, and hence, functional characteristics of different aged animals. The response of the adult myocardium to increased levels of O_2 present in CP solutions, and during reperfusion, may be explained by the distribution of saturated fatty acids within the phospholipid matrices of cellular membranes. Yam and associates[38] examined the effects of hyperoxia on adult and neonatal rat lungs.[38] They found that the neonates have an increased resistance to oxygen induced lung damage, which may be caused by a rapid increase in endogenous antioxidants (superoxide dismutase and glutathione) which reduce FOR injury.

The decrease in hemodynamic recovery seen in groups receiving oxygenated cardioplegia may not reflect alterations in membrane permeability, which is a characteristic finding in damaged myocytes. During ischemia CK washout increased in both adult and neonatal low dose oxygen groups, indicating the more permeable state of the sarcolemma. Upon reperfusion

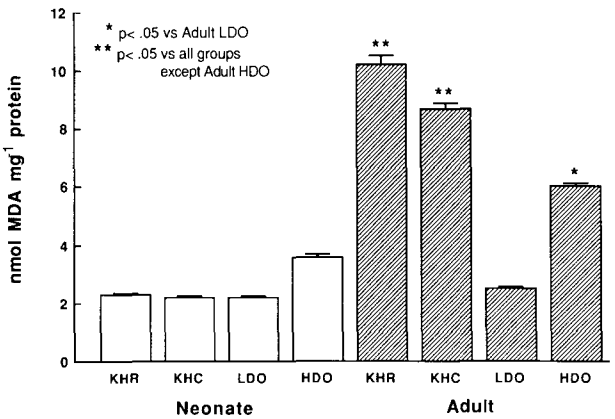

Figure 6: Whole heart homogenate malondialdehyde (MDA) content. KHR, Krebs-Henseleit with reperfusion. KHC, Krebs-Henseleit biochemical control. LDO, low dose oxygen. HDO, high dose oxygen. n = 6 for each group. All data are mean ± SEM.

CK returned to near baseline levels across all cardioplegic treated groups, while the Krebs-Henseleit neonate group was greatly elevated. Several investigators have described a pathological state in which the postischemic recovery of myocardial function was depressed in the early reperfusion period, which was not a result of myocardial necrosis.[39,40] They suggest that postischemic myocardial structural, functional, and metabolic properties are depressed below preischemic values, although irreversible cellular injury may not be detected. This has been termed the "stunned myocardium" and may limit the initial recovery of the heart. The toxic action of FOR has been implicated in causing this myocardial depression, and the damage may be exacerbated by the washout of free oxygen radical scavengers from previously ischemic tissue.[41] The replenishment of free oxygen radical scavengers following brief periods of coronary occlusion in dog hearts resulted in reduced occurrence of factors implicated in the "stunned myocardium" phenomenon.[40]

The coronary circulation responds to periods of hypoxia with vasodilatation, which is an attempt to reduce the substrate deficit incurred by oxygen deprivation. This autoregulatory mechanism results in decreased coronary vascular resistance and is termed reactive hyperemia. A potent vasoactive substance is the nucleoside adenosine which is generated by the hydrolysis of ATP during ischemia. Although our study did not measure adenosine concentrations, indirect evidence may indicate an enhanced susceptibility of the neonatal coronary circulation to autoregulatory effects in the early postischemic time period. These responses were highlighted in all neonatal hearts, as evidenced by large increases in postischemic coronary flow. Newborn hearts are known to contain a greater quantity of capillaries per unit mass of heart tissue with the capillary to fiber ratio approaching 6 to 1, while the mature heart is closer to 1 to 1.[41] This increased vasculature may represent a means by which the newborn increases oxygen and substrate delivery, enhancing metabolic recovery by the rapid production of high energy phosphates. These substances may then be channeled into reparative mechanisms of cellular membranes which preempt the functional recovery of the heart. Such reperfusion alterations offer attractive theories in explaining the neonates' known resistance to hypoxic and ischemic injury and deserve further exploration.

Hyperoxygenation of crystalloid CP solutions can potentially result in both the generation of FOR as well as an increased risk of gaseous microembolization. Although gross emboli would have been trapped in the inline bubble trap, no effort was made to determine the existence of microbubbles which may have vasoactive effects on the coronary circulation. The ultimate response is a similar disruption of cellular membranes either by the cytotoxic effects of FOR or the generation of ischemic zones in the myocardium.

In conclusion, our results question the advantages of utilizing oxygenated crystalloid cardiolegic solutions to protect the myocardium during moderate hypothermic ischemia. We were unable to show substantial improvement in recovery when an oxygenated cardioplegic solution was utilized. In adult hearts a clear advantage of cardioplegic administration was evident, while in neonates the hemodynamic differences were not as pronounced. Furthermore, the generation of lipid peroxidation products was correlated with high oxygen tensions in adult hearts, but was not as evident in the newborn. This supports previous studies that describe the efficacy of free oxygen radical scavengers in reducing reperfusion related injury in the mature myocardium. However, their effectiveness as antioxidants in the newborn myocardium remains questionable.

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Questions from the Audience

Question: Aaron Hill, Falls Church, VA: Very nice presentation. I saw what appeared to me some conflicting data—high dose and low dose oxygen. Where the animals recovered well the malondialdehyde studies would not have predicted that. Would you agree from the data?

Response: I think something similar to that I did recognize and that was mainly with creatine kinase. The low dose oxygen had greater quantities of creatine kinase that were released during the ischemic period, although significance wasn't met. However, malondialdehyde, I believe, is a purer indicator of the actual cellular injury in this particular model. Creatine kinase, like ATP, I think, has not been 100% correlated with the ability of the heart to recover following an ischemic interval. I think our data here supports that. Again, looking at the actual cellular membranes themselves, maybe they're a more finely tuned indicator of that type of entry. So you're right. There is a sort of paradox here. It would be nice if they did follow the same correlation. The only way I can answer that is perhaps, again in that fashion, MDA will be more definitive.

Question: It would appear that when looking at the glycolytic pathway mechanism, as opposed to the membrane stabilization mechanism, that your data from the reperfusion aspect would probably support

the glycolytic pathway as opposed to the other method that the neonates used to protect themselves. And neonates protect themselves that way and not through membrane stabilization. Would you agree?
Response: That's a very good point. In fact, glycolysis has been shown to be probably the major area of myocardial preservation employed by the neonates and in the adults. And glycolysis is basically shut off during the build-up of certain metabolites that are produced during ischemia and knocking out both phospholipid molecule and linolenic and arachiodonic acid in the adult myocardium. However, the neonates have been shown to be more tolerant because the same suppression does not occur across these two molecules. Also, along the same lines neonates are closer in their artrogenic development to anaerobic respiration that they've seen through their intrauterine development. They don't have high quantities of oxygen available to them and their respiratory function has, therefore, not been as enhanced as it is in the adult environment.
Comment/Question: Richard Berryessa, Denver, CO: Nice study supported in part, if I recall correctly, by a research grant from AmSECT. I have two questions. One asked for you to look at the ideology of the difference between the neonatal and the adult heart—by looking partly at the mechanism for generating free oxygen species. The Embden-Meyerhoff pathway is only one pathway. And the generation of free radicals by the breakdown of the prostaglandin and the peroxides. I wonder because of the difference in the lipid makeup of cell membranes—higher incidence of linoleic acid is supposed to be arachidonic acid in the infant hearts. If that offers some protection because of the decreased production of Thromboxane A_2 a decrease in arachidonic acid and the increase in linoleic acid. The second question: Is it possible because of the difference in the polyunsaturated fats in with the membranes to perhaps protect adult and maybe even infant hearts by somehow modifying the diet or the makeup of the cell membrane, making them more tolerant to ischemia? Maybe they would even be more cold tolerant because that is really the difference between cold tolerant animals and animals that are not—the degree of polyunsaturated fats in their cell membranes. Maybe this could be done somehow modifying and changing their arachidonic acid or linolenic acid by giving them cod liver oil or some other crazy thing. What do you think?
Response: Good points, Richard. Just to make one correction: the linolenic and arachidonic polyunsaturated fatty acids were of lower quantities in the neonates here so they were basically showing a decreased susceptibility. Neutrophil mediation in regard to free oxygen radical damage proceeds by basically vascular changes in the epithelium and more so on an extracellular level. Also, the alteration of membrane systems by diet manipulation is surely something where we can use exogenous antioxidants such as Vitamin E or any of the fat soluble vitamins to get right inside of the cellular matrices and stop the propagation in the chain reaction that we have shown in that one slide. I'm not pushing megavitamin therapy, although *Newsweek* surely would definitely go along those lines. If you could alter your exogenous antioxidant diet maybe we would reduce this type of injury which is basically what some people believe is what is caused by aging. It is just an accumulation of the toxic oxygen metabolites over many years. So I don't know. That's a good point.